NEOPLASIA
www.neoplasia.com

Volume 13 Number 5 May 2011 pp. 472–482 472

p38γ Mitogen-Activated Protein Kinase Contributes to Oncogenic Properties Maintenance and Resistance to Poly (ADP-Ribose)-Polymerase-1 Inhibition in Breast Cancer[1,2]

Fanyan Meng[*,†], Haijun Zhang[*,†], Gang Liu[*,†], Bas Kreike[‡], Wei Chen[§], Seema Sethi[¶], Fred R. Miller[*,†] and Guojun Wu[*,†]

*The Breast Cancer Biology Program, Barbara Ann Karmanos Cancer Institute, Detroit, MI, USA; [†]Department of Oncology and Pathology, Wayne State University School of Medicine, Detroit, MI, USA; [‡]Division of Radiation Oncology and Experimental Therapy, the Netherlands Cancer Institute, Amsterdam, The Netherlands; [§]Biostatistics Core, Karmanos Cancer Institute, Department of Internal Medicine, Wayne State University School of Medicine, Detroit, MI, USA; [¶]Karmanos Cancer Institute Biorepository Core, Department of Pathology, Wayne State University School of Medicine, Detroit, MI, USA

Abstract

p38γ MAPK, one of the four members of p38 mitogen-activated protein kinases (MAPKs), has previously been shown to harbor oncogenic functions. However, the biologic function of p38γ MAPK in breast cancer has not been well defined. In this study, we have shown that p38γ MAPK is overexpressed in highly metastatic human and mouse breast cancer cell lines and p38γ MAPK expression is preferentially associated with basal-like and metastatic phenotypes of breast tumor samples. Ectopic expression of p38γ MAPK did not lead to an increase in oncogenic properties *in vitro* in most tested mammary epithelial cells. However, knockdown of p38γ MAPK expression resulted in a dramatic decrease in cell proliferation, colony formation, cell migration, invasion *in vitro* and significant retardation of tumorigenesis, and long-distance metastasis to the lungs *in vivo.* Moreover, knockdown of p38γ MAPK triggered the activation of AKT signaling. Inhibition of this feedback loop with various PI3K/AKT signaling inhibitors facilitated the effect of targeting p38γ MAPK. We further found that overexpression of p38γ MAPK did not promote cell resistance to chemotherapeutic agents doxorubicin and paclitaxel but significantly increased cell resistance to PJ-34, a DNA damage agent poly (ADP-ribose)-polymerase-1 (PARP) inhibitor *in vitro* and *in vivo*. Finally, we identified that p38γ MAPK overexpression led to marked cell cycle arrest in G_2/M phase. Our study for the first time clearly demonstrates that p38γ MAPK is a promising target for the design of targeted therapies for basal-like breast cancer with metastatic characteristics and for overcoming potential resistance against the PARP inhibitor.

Neoplasia (2011) 13, 472–482

Abbreviations: MAPK, mitogen-activated protein kinase; PARP, poly (ADP-ribose)-polymerase-1; Dox, doxorubicin; Pac, paclitaxel; HR, homologous recombination; ERK, extracellular signal–regulated kinase

Address all correspondence to: Guojun Wu, PhD, Breast Cancer Biology Program, Karmanos Cancer Institute, Departments of Oncology and Pathology, Wayne State University School of Medicine, HWCRC, Room 840.2, 4100 John R, Detroit, MI 48201. E-mail: wugu@karmanos.org

[1]This work was supported in part by the Susan G. Komen grant KG080465 (G. Wu) and a pilot grant from the Karmanos Cancer Institute, Wayne State University School of Medicine (G.W.). The authors declare that they have no competing interests.

[2]This article refers to supplementary materials, which are designated by Figures W1 to W15 and are available online at http://www.neoplasia.com.

Received 22 December 2010; Revised 15 February 2011; Accepted 18 February 2011

DOI 10.1593/neo.101748

Introduction

The p38 group of kinases belongs to the mitogen-activated protein kinase (MAPKs) superfamily and has structural and functional characteristics distinguishable from those of the extracellular signal–regulated kinase (ERK) and c-Jun N-terminal kinases (JNK) [1,2]. There are four members of the mammalian p38 mitogen-activated protein kinases (p38α, p38β, p38γ, and p38δ). These MAPKs are approximately 60% identical in their amino acid sequences but differ in their expression patterns, substrate specificities, and sensitivities to chemical inhibitors [3–7]. All p38 MAPKs are strongly activated *in vivo* by environmental stresses and inflammatory cytokines [1,8,9]. However, recent studies indicate that the physiological functions of different members of the p38 MAPKs may overlap but not necessarily be identical and/or redundant [10–12].

The distinct biologic role of p38γ (also named MAPK12, ERK6, and SAPK3) has been explored in recent years. In contrast to p38α, which has been shown ubiquitously expressed, p38γ is only detectable in certain normal tissues including skeletal muscle [3,13], and it was also found to be highly expressed in several human cancer cell lines [14–17]. In addition, activation of the p38γ MAPK cascade—though no other p38 isoforms—is sufficient to induce G_2 arrest in cells. Activation of p38γ is dependent on ATM and leads to the activation of Cds1 (also known as Chk2). These data suggest a model in which activation of ATM by γ irradiation leads to the activation of MKK6, p38γ, and Cds1 and that activation of both MKK6 and p38γ is essential for the proper regulation of the G_2 checkpoint in mammalian cells [17]. Particularly, overexpression of Rit, a Ras family member, in NIH3T3 cells, causes transformation and stimulation of the expression of p38γ but not other p38 MAPKs, ERK1/2, or ERK5 [18]. Consistent with this finding, several studies suggest that the Ras oncogene positively regulates the expression of p38γ and depletion of p38γ suppressed Ras transformation in rat intestinal epithelial cells [16]. Interestingly, p38γ also integrates into the crosstalk between RAS and ER and increases breast cancer invasion [19]. It has also shown that p38γ MAPK cooperates with c-JUN in transactivating MMP9 [20]. Specifically, p38α and p38γ mediate oncogenic *ras*-induced senescence through differential mechanisms [21]. Although the above data strongly indicate the possible oncogenic role of p38γ MAPK in contributing to cellular transformation and invasive phenotype, the precise functional role of p38γ MAPK in breast oncogenesis and drug resistance has not been investigated.

In this study, we specifically investigated the expression of p38γ MAPK in breast cancer cell and tumor samples. We also characterized the biologic effect of ectopic expression and knockdown expression of p38γ MAPK on breast cancer cell oncogenic properties including the cell proliferation, colony formation, cell migration and invasion *in vitro*, and tumorigenesis and metastasis *in vivo*. The effect of p38γ MAPK on cell signaling was also investigated, and a signaling feedback loop caused by knockdown of p38γ MAPK was identified. Finally, we assessed the significance of p38γ MAPK expression on cell sensitivity to various drug treatments, including chemotherapeutic agents doxorubicin (Dox) and paclitaxel (Pac) and the DNA damage agent poly (ADP-ribose)-polymerase-1 (PARP) inhibitor PJ-34. Our study represents the first comprehensive investigation of the biologic function of p38γ MAPK in breast cancer. The results will facilitate the development of anti–breast cancer therapeutics based on molecular targeting of p38γ MAPK.

Materials and Methods

Cell Cultures

Mouse breast cancer cell lines 4T1, 4T07, 168FARN, and 67 NR were originally generated by one of the authors (F.R.M.) and have been characterized for their metastasis capability *in vivo* [22]. These cells were cultured in high-glucose Dulbecco modified Eagle medium supplemented with 5% fetal bovine serum, 5% newborn calf serum (NCS), nonessential amino acids (NEAA), and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). All human breast cancer cell lines were obtained from and characterized by cytogenetic analysis by American Type Culture Collection (ATCC, Manassas, VA). All these cells were grown per ATCC recommendations. The mouse mammary epithelial cell line EpRas and the human mammary epithelial cell line HMLE were obtained from Robert A. Weinberg's laboratory at Massachusetts Institute of Technology. EpRas was maintained in Dulbecco modified Eagle medium, 8% fetal bovine serum, and 500 μg/ml G418. HMLE was maintained in the culture as described [23]. Both cell lines were authenticated on receipt by comparing them to the original morphologic and growth characteristics [24].

Expression Constructor, Short Hairpin RNA, and Lentivirus Generation

A full-length p38γ MAPK plasmid was purchased from Open Biosystems (Huntsville, AL). A set of short hairpin RNA (shRNA) clones for p38γ MAPK gene was purchased from Open Biosystems. The generation of different lentivirus and stable cell lines was according to standard procedures [25].

Reverse Transcription–Polymerase Chain Reaction and Western Blot

Reverse transcription–polymerase chain reaction (RT-PCR) and Western blot analysis were performed as previously described [25,26]. p38γ MAPK polycolony antibody (R&D Systems, Minneapolis, MN) was used for Western blot analysis.

Transwell Migration and Invasion Assays

Cell migration and invasion assays were performed as before [25,26].

Tissue Microarray and Immunohistochemistry Assay

A breast cancer tissue array, 150 cores including 75 cases of normal, reactive, premalignant, and malignant tissues of the breast in duplicates, was purchased from Pantomics (San Francisco, CA). Standard procedures were used for immunohistochemistry assay. The p38γ MAPK antibody was purchased from Origene (Rockville, MD), and the working dilution is 1:50.

In Vivo *Metastasis Assay*

The MCF7-derived tumor cells (1×10^7) in 100 μl of PBS and Matrigel were injected into the mammary gland of 5-week-old female NCR *nu/nu* mice (Taconics, Hudson, NY). The 4T1-derived tumor cells (2×10^4) in 50 μl of PBS were injected into the mammary gland of 5-week-old female BALB/C mice (Jackson Laboratory, Bar Harbor, ME). After tumor cell injections, mice were killed at the fourth week. The mammary tumor and lungs were removed and embedded into paraffin blocks. Standard hematoxylin and eosin staining of paraffin-embedded tissue was performed for histologic examination of metastases.

Chemoresistance Assay, Cell Proliferation, and Colony Formation Assay

Cells with p38γ MAPK overexpression or knockdown and their vector control were seeded into a 48-well or a 96-well plate (1×10^3 or 5×10^3 cells/well). After 24 hours, the cells were treated with chemotherapy agents (Dox and Pac) and PARP inhibitor PJ-34 for 24 hours with

the indicated doses. After being cultured in a drug-free growth medium for another 48 hours, the surviving cells were quantified by MTT assay or clonogenic assay. An MTT assay was performed as described in the protocol (Promega, Madison, WI) [26]. For the clonogenic assay, the harvested cells were seeded onto a 10-cm dish. Crystal violet staining was used to count the colonies after a 10-day culture in growth medium. The untreated cells were used to normalize the experimental data.

Cell Cycle Analysis

Cells were seeded in a six-well plate and PJ-34 was added to the cells after 24 hours. At the indicated times, aliquots of 2×10^5 cells were washed with PBS and fixed with 70% (vol/vol) ethanol dropwise for 4 hours at −20°C. Cells were washed with PBS, treated with RNAse A (1 mg/ml) for 30 minutes at 37°C, and stained with propidium iodide (0.05 mg/ml) for 10 minutes at room temperature. Samples were analyzed using a FACScan flow cytometer.

Statistical Analysis

A two-sided independent Student's *t* test without equal variance assumption was performed to analyze the results of cell growth, colony formation, cell migration and invasion, luciferase assay, tumor burdens, and tumor metastasis. The Cochran-Armitage test was used to analyze the statistical significant linear trend between the expression of p38γ MAPK and metastasis phenotypes.

Results

p38γ MAPK Expression Is Associated with Aggressive Basal-like Breast Cancer and Metastatic Phenotype in Breast Tumor Samples

To investigate the p38γ MAPK expression pattern in breast cancer, we first determined the expression of p38γ MAPK in a set of human breast cancer cell lines with different metastatic capabilities. We found that a high expression of p38γ MAPK was shown in basal-like and highly metastatic cancer cell lines, whereas a low expression of p38γ MAPK was seen in most nonmetastatic or poorly metastatic cell lines (Figure 1*A*, *top panel*). In addition, we found that p38γ MAPK protein levels are perfectly consistent with RNA levels, which are higher in highly metastatic cell lines compared with nonmetastatic or poorly metastatic cell lines (Figure 1*A*, *low panel*). Moreover, p38γ MAPK expression was also studied using a panel of mouse model cell lines. Among these four cell lines, 4T1 is the most highly metastatic cell line and can metastasize spontaneously from mammary fat pads to the lungs, liver, and bone (long bones and vertebrae) in most animals. 4T1 also metastasizes to the brain and adrenal gland in some animals, thus reproducing the pattern found clinically in human breast cancer. The three remaining cell lines are nonmetastatic subpopulations that fail to metastasize at different points in dissemination. 67NR fails to leave the primary site, 168FARN spreads to the lymph nodes but travels no further, and 4TO7 cells are routinely recovered from the

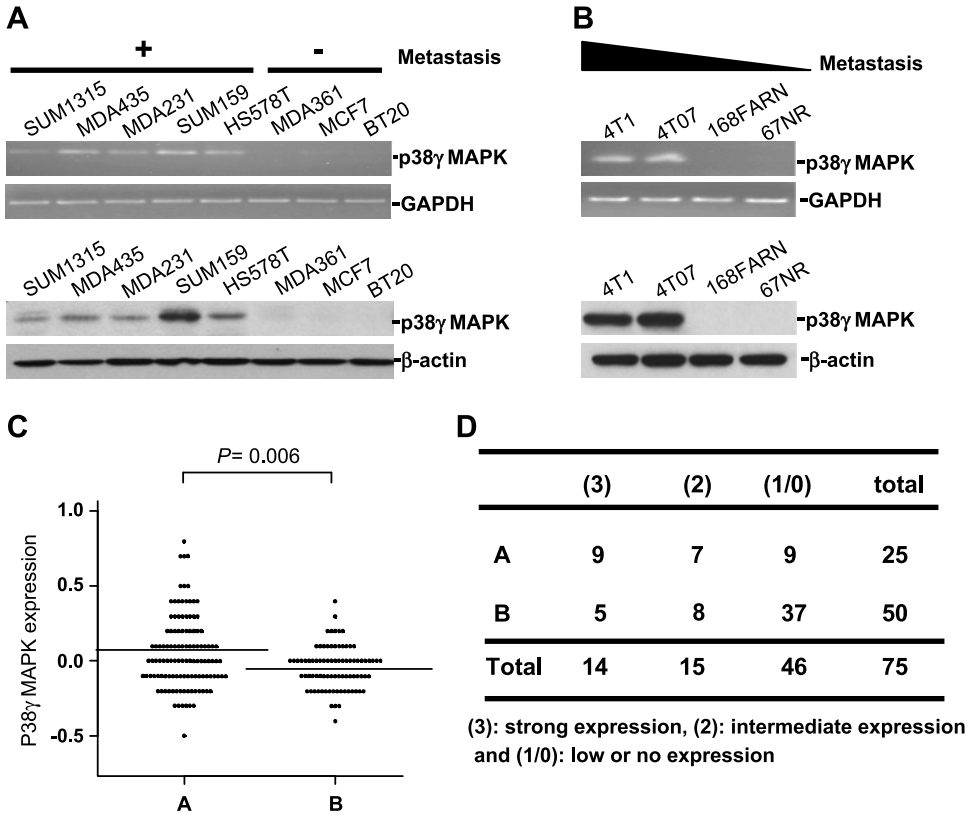


Figure 1. Expression of p38γ MAPK in breast cancer cell lines and breast tumor tissues. (A and B) Expression of p38γ MAPK in human (A) and mouse (B) highly metastatic cell lines. Top panel shows RT-PCR results. Lower panel shows results of Western blot analysis. (C) Expression of p38γ MAPK is preferentially associated with basal-like breast cancer ($P = .006$). Group A is basal-like breast tumor (116 cases); group B is non–basal-like breast tumor (83 cases). (D) IHC assay shows that p38γ MAPK is significantly overexpressed in the tumor samples with lymph node and distant metastasis ($P = .001$). Group A ($n = 25$) includes tumor samples with lymph node and distant metastasis. Group B ($n = 50$) includes tumor samples without metastasis and normal tissue.

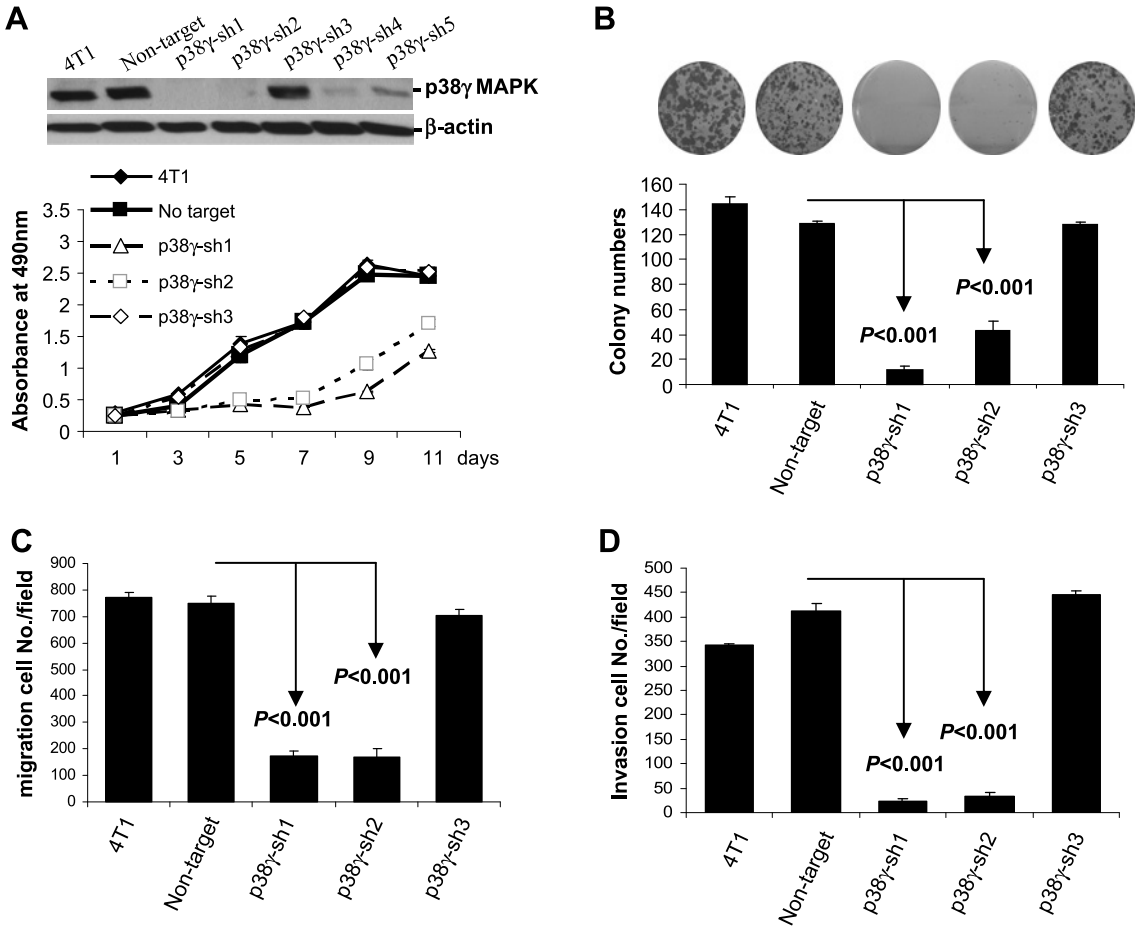


Figure 2. Effect of knockdown of p38γ MAPK on oncogenic properties *in vitro*. (A) Knockdown of p38γ MAPK expression in 4T1 cells significantly inhibits cell proliferation. Top panel shows five shRNA clones that differentially inhibit the expression of p38γ MAPK in 4T1 cells. β-Actin is a protein-loading control. Lower panel shows two clones with knockdown of p38γ MAPK expression that have inhibited cell proliferation compared with parental 4T1 cell and 4T1 with nontarget control in 7 days. (B, C, and D) Knockdown of p38γ MAPK expression in 4T1 cells significantly inhibits colony formation (B), cell migration (C), and invasion (D). Two clones (sh1 and sh2) with knockdown of p38γ MAPK expression and one clone (sh3) without significant p38γ MAPK expression change were used to compare with parental 4T1 cell and 4T1 with nontarget control.

lungs but fail to proliferate [22]. The expression level of p38γ MAPK in mouse model cell lines was found to be concomitant with the metastatic capability as shown by RT-PCR and Western blot (Figure 1*B*). In contrast to p38γ MAPK, the expression of three other p38 MAPKs including p38α, p38β, and p38δ MAPK showed no consistent correlation to basal-like and metastatic phenotype in both human and mouse breast cancer model cell lines (Figure W1).

We then sought to determine whether the expression of p38γ MAPK correlates with certain pathologic phenotypes in clinical breast tumor samples. We analyzed a breast cancer gene expression data set, which contained 116 basal-like breast tumors and 83 non–basal-like breast tumors according to their triple-negative status. We found that the level of p38γ MAPK expression was significantly higher in basal-like breast tumors than in non–basal-like breast tumors ($P = .006$) (Figure 1*C*). Moreover, we performed an immunohistochemistry (IHC) to investigate the p38γ MAPK expression in breast tumors using a tissue microarray. The relation of p38γ MAPK and pathologic characteristics was analyzed (Figure W2*B*). Specifically, we found that high expression of p38γ MAPK is present in 36% of the breast tumors with metastasis and in only 10% of the breast tumors without metastasis). In contrast, a low expression of p38γ MAPK was found in only 36% of breast tumors with metastasis and in 74% of breast tumors without metastasis (Figures 1*D* and W2*A*). The Cochran-Armitage test for trend reveals that there is a statistically significant linear trend between the expression of p38γ MAPK gene and metastatic phenotypes ($P < .001$). Overall, these results suggest that the high expression of p38γ MAPK is a molecular occurrence related to the metastatic phenotype and basal-like characteristics in breast cancer cell lines and tumor samples.

Knockdown of p38γ MAPK Significantly Inhibits Oncogenic Properties In Vitro *and* In Vivo

To investigate the function of p38γ MAPK in breast cancer oncogenic properties, we first established knockdown model cell lines based on the highly metastatic mouse mammary epithelial cell 4T1, which also shows the most predominant p38γ MAPK expression. When compared with parental 4T1 and 4T1 cells with nontarget control and vector control, three of five shRNA clones showed more than 80% shut down of p38γ MAPK expression as evidenced by the Western blot (Figure 2*A*). We further observed that inhibition of p38γ MAPK expression markedly inhibits the cell proliferation and colony formation (shRNA clone1/nontarget control = 80%, $P < .001$ and shRNA clone2/nontarget control = 68%, $P < .001$) using these stable cell lines

(Figures 2*A*, W3*A*, and 2*B*). Moreover, knockdown of p38γ MAPK expression in 4T1 cells led to a marked decrease in cell migration (80%, $P < .001$) (Figures 2*C* and W3*B*) and invasion (93.75%, $P < .001$) (Figures 2*D* and W3*B*).

We then tested the effect of p38γ MAPK knockdown on breast cancer tumorigenesis and metastasis *in vivo* using BALB/C mice after orthotopic injection. Cells with two p38γ MAPK knockdown shRNA clones (shRNA1 and shRNA2) showed significant retardation in tumor burden in the mammary gland after 28 days compared with cells with nontarget control, shRNA3 and parental 4T1 cells (Figure 3*A*). Cells with the shRNA1 clone showed smaller tumor burden compared to cells with the shRNA2 clone, suggesting a correlation of p38γ MAPK expression with tumorigenesis, data consistent with the result of the *in vitro* study. Moreover, significantly reduced lung metastasis was also observed in mice implanted with cells of two p38γ MAPK knockdown shRNA, although cells with nontarget control showed similar quantities of lung metastasis to parental 4T1 cells (Figure 3, *B* and *C*). Taken together, these data suggest that a high-level expression of p38γ MAPK is critical for breast cancer cells to maintain their oncogenic properties *in vitro* and *in vivo*.

Ectopic Expression of p38γ MAPK Alone Cannot Promote Oncogenic Properties In Vitro *and* In Vivo

To complement the results we obtained above, we generated p38γ MAPK ectopic expression models based on five different breast cancer cell lines using a lentivirus system. These cell lines are BT20, MCF7, HMLE, 67NR, and EpRas, and they all have low or no expression of p38γ MAPK. We then investigated the effect of p38γ MAPK ectopic expression on oncogenic characteristics. Interestingly, we did not observe any significant change in cell proliferation, colony formation, cell migration, and invasion in most of the tested cell lines except MCF7 (Figures 4, W5, W6, W7, and W8). The MCF7 cell line with ectopic expression of p38γ MAPK had no change in cell proliferation and

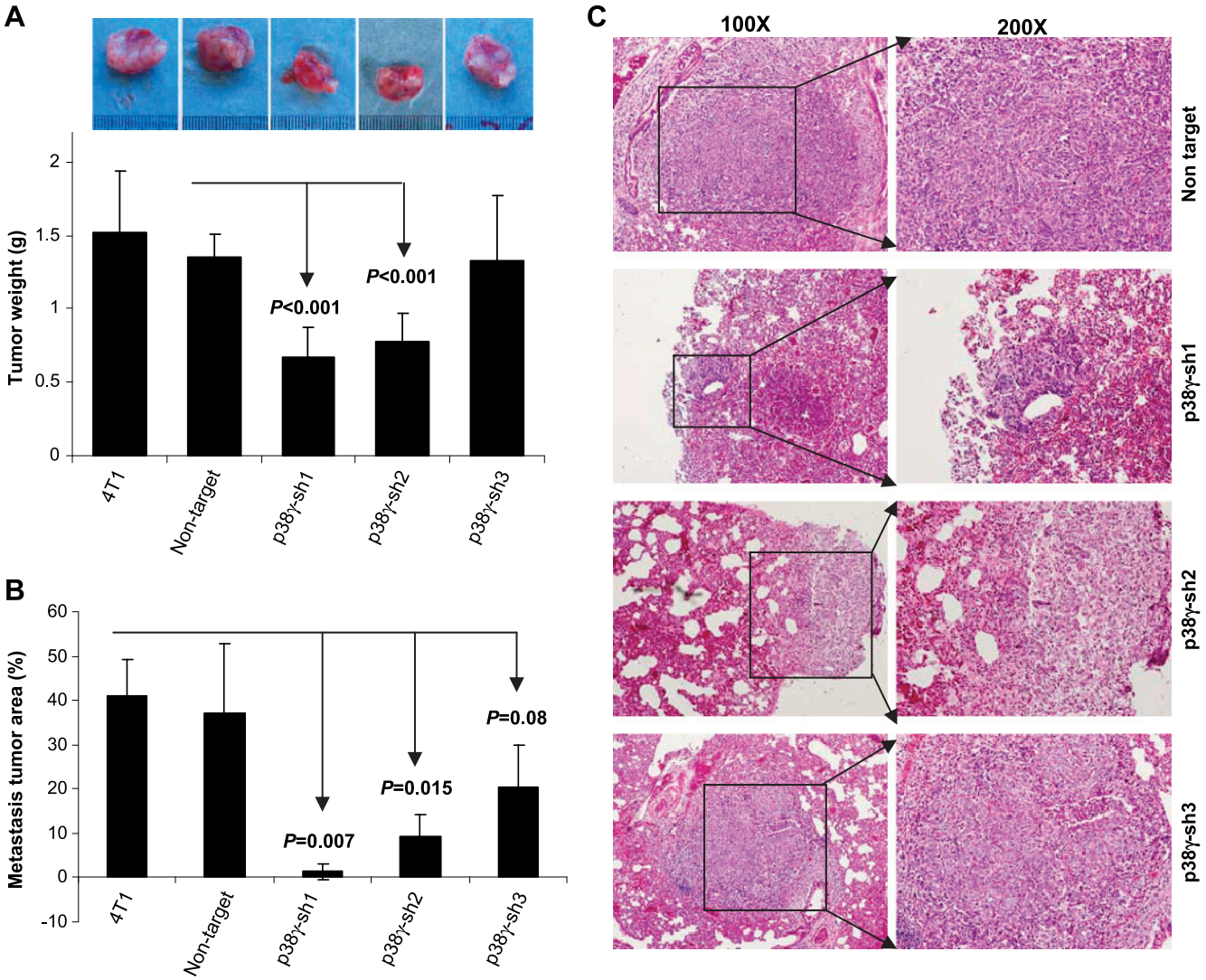


Figure 3. Effect of knockdown of p38γ MAPK expression in tumorigenesis and distal metastasis *in vivo*. (A and B) Two clones with knockdown p38γ MAPK expression (p38γ-sh1 and p38γ-sh2) show inhibited tumor burden *in vivo* (A) and reduced lung metastasis (B) compared with parental 4T1 cell, 4T1 with nontarget control, and 4T1 cells without significant p38γ MAPK expression change (p38γ-sh3). Ten mice were used in each group. (C) Representative hematoxylin and eosin staining pictures of lung sections from mice injected with 4T1-derived cells (magnification, ×100 and ×200). The origin of the lung section is shown on the right of the panels.

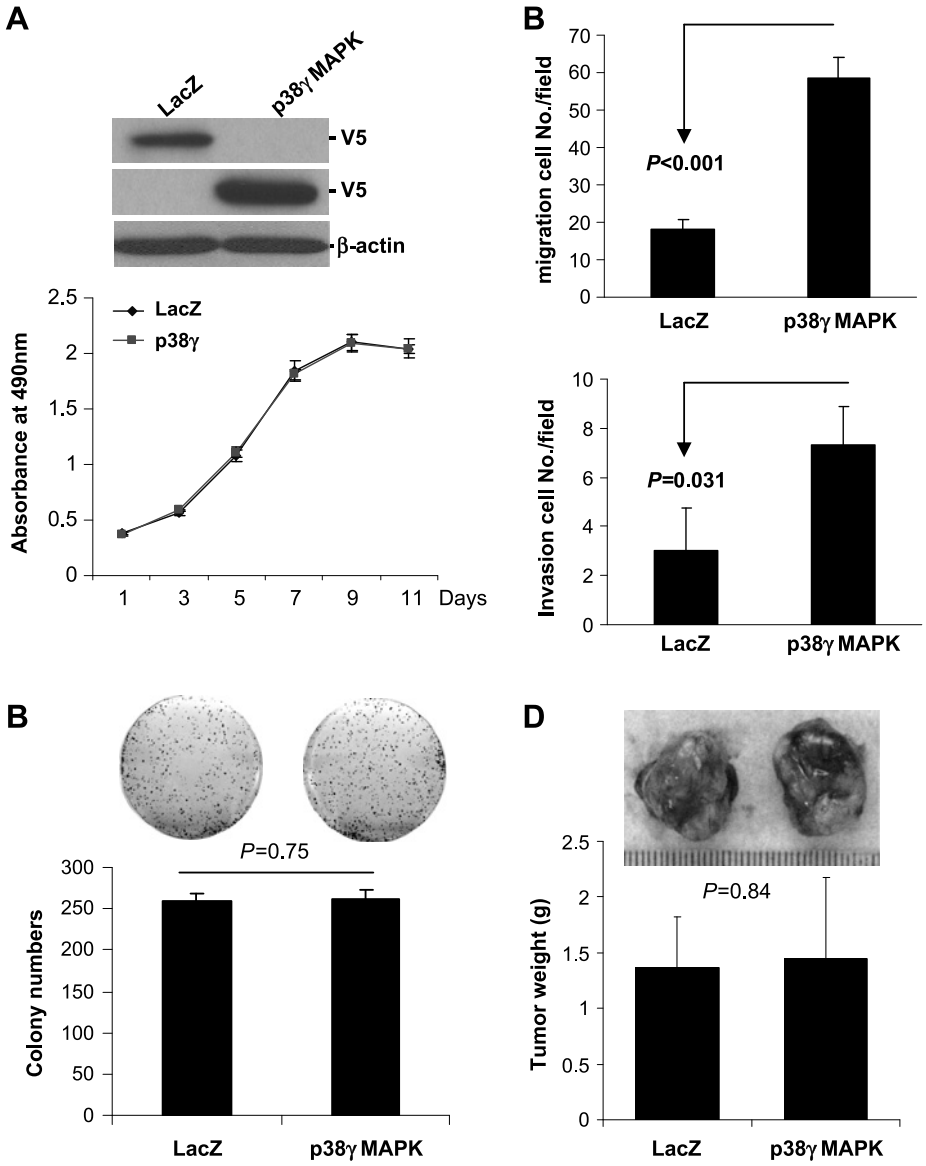


Figure 4. Effect of ectopic expression of p38γ MAPK on oncogenic properties of MCF7 cells *in vitro* and *in vivo*. (A) Ectopic expression of p38γ MAPK in MCF7 cells has no effect on cell proliferation. The top panel shows ectopic expression of p38γ MAPK in MCF7 cells. V5 antibody is used to detect p38γ MAPK. β-actin is a protein loading control. The lower panel shows cell proliferation of MCF7 cells expressing vector control and p38γ MAPK gene in 11 days. (B) Ectopic expression of p38γ MAPK in MCF7 cells does not lead to change in colony formation compared with MCF7 cells with vector control. (C) Ectopic expression of p38γ MAPK in MCF7 cells leads to an increase in cell migration (top panel) and cell invasion (low panel) compared with MCF7 cells with vector control. (D) Effect of ectopic expression of p38γ MAPK on tumorigenesis. The cells with ectopic p38γ MAPK expression show no significant difference in tumor burden *in vivo* compared with parental MCF7 cell with vector control. Eight mice were used in each group.

colony formation but had a increase in cell migration (3-folds, $P < .001$) and invasion (2.5-folds, $P = .031$) compared with their vector control counterparts (Figures 4*C* and W4, *A* and *B*).

To further validate the role of p38γ MAPK in oncogenesis, we orthotopically injected the MCF7 model cell line with ectopic expression of p38γ MAPK into the fat pads of nude mice and tested for the tumorigenesis and metastatic propensity. Ectopic expression of p38γ MAPK in MCF7 showed no difference in tumor growth speed and final tumor burden compared with MCF7 cells with vector control (Figure 4*D*). Similarly, no significant difference in lung metastasis was observed in MCF7 cells with and without p38γ MAPK expression (Figure W4*C*). Taken together, these data suggest that overexpression of p38γ MAPK alone is insufficient to promote tumorigenesis and metastasis.

Knockdown of p38γ MAPK Leads to a Feedback Signaling Loop

To explore the mechanism underlying p38γ MAPK function, we checked the common cell signaling pathways related to cell proliferation, mobility, and cell-to-cell interaction. Western blot results showed that knockdown of p38γ MAPK in 4T1 significantly decreased expression of p-ERK. The result of cell signaling is concomitant with decreased cell proliferation, cell invasion and migration, and retarded tumorigenesis and inhibited long-distance metastasis. Interestingly, p-AKT and p-Src expression was significantly increased because of the knockdown of p38γ MAPK (Figure 5*A*). Conversely, overexpression of p38γ MAPK led to a decrease in p-AKT in both MCF7 and EpRas cells and an increase in p-ERK in only EpRas cells (Figure W9).

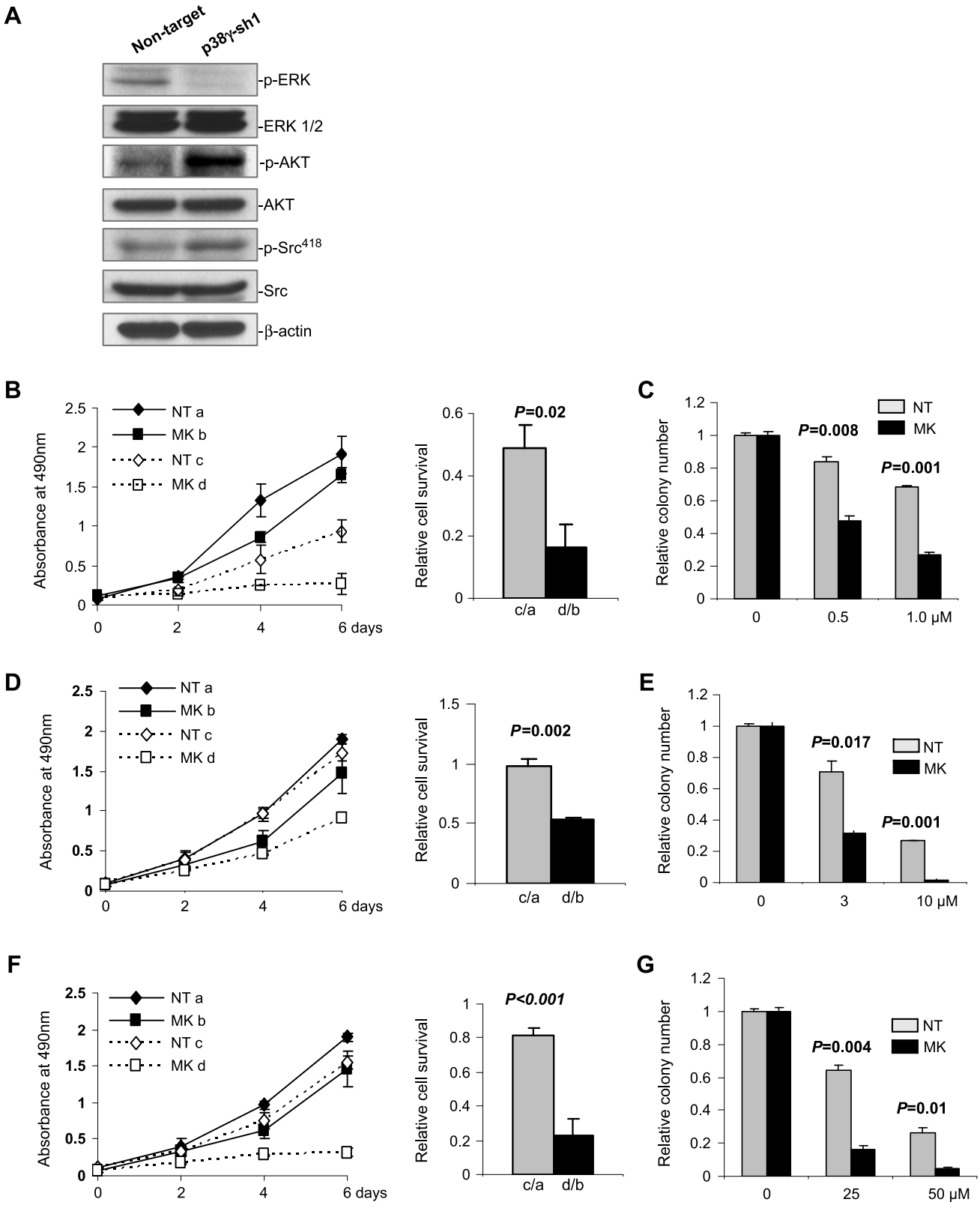


Figure 5. Effect of p38γ MAPK on cell signaling pathways. (A) Effect of p38γ MAPK on AKT and ERK signaling by Western blot analysis assay. Western blots show the changes in cell signaling when p38γ MAPK is knocked down in 4T1 cells. (B, D, and F) The effect of combination of knockdown of p38γ MAPK and various AKT signaling inhibitors on cell proliferation with different time courses. The right panels show the final comparison of the cell survival rate between nontarget clone and knockdown clone after treatment of different inhibitors. (C, E, and G) Effect of the combination of knockdown of p38γ MAPK and various AKT signaling inhibitors on colony formation with different doses. 4T1 cells with p38γ MAPK knockdown (MK) and nontarget clone (NT) were treated with AKT inhibitor (AKti-1/2) (B and C), PI3K inhibitor (LY294002) (D and E), and Src inhibitor (Dasatinib) (F and G).

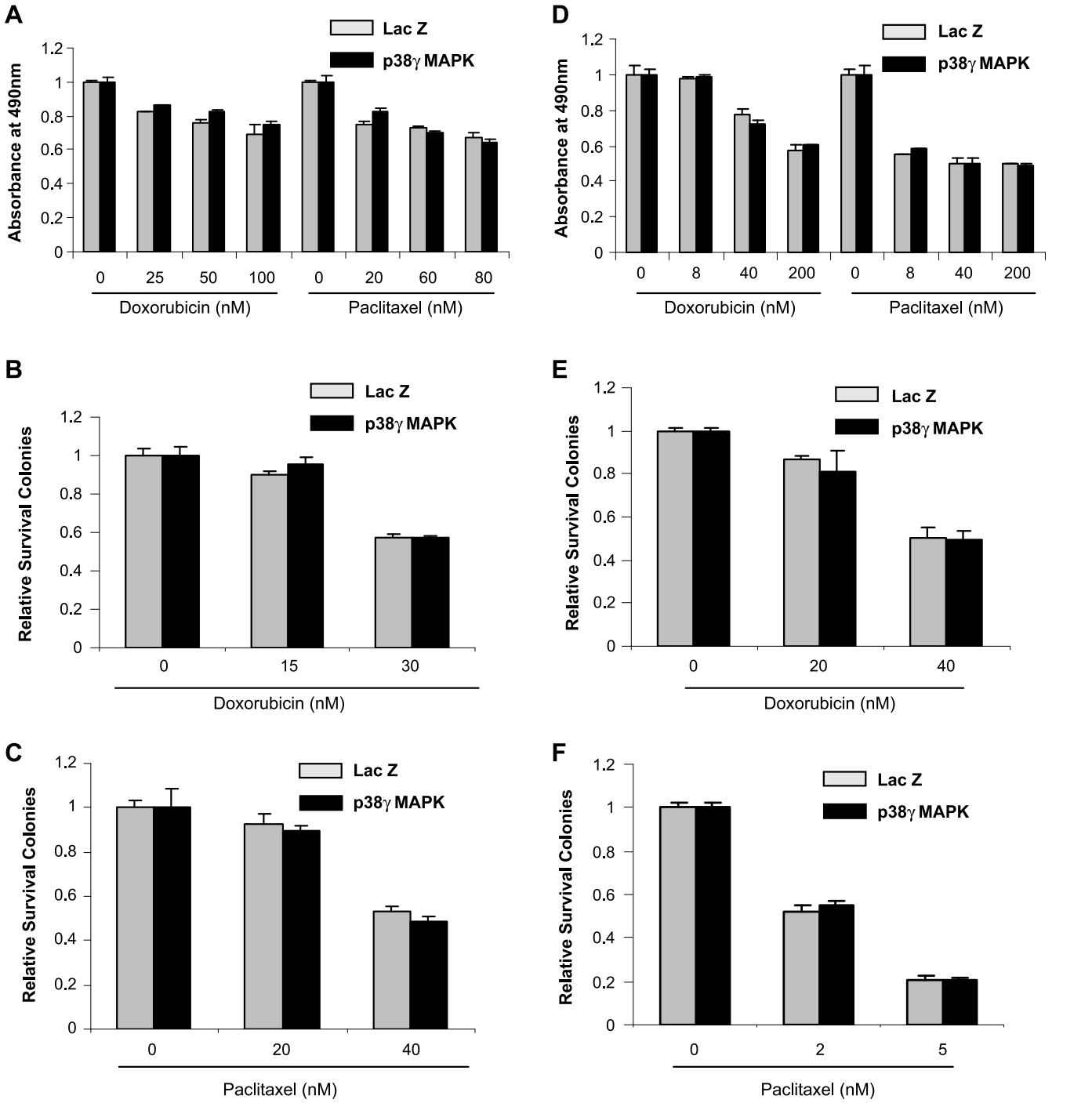


Figure 6. Effects of Dox and Pac on 67NR and MCF7 cells with or without p38γ MAPK overexpression. (A and D) Overexpression of p38γ MAPK in 67NR (A) and MCF7 (D) cells has no effect on the cell proliferation under the treatments with Dox and Pac. (B and E) Overexpression of p38γ MAPK in 67NR (B) and MCF7 (E) cells has no effect on the colony formation capability under the treatments with Dox. (C and F) Overexpression of p38γ MAPK in 67NR (C) and MCF7 (F) cells has no effect to the colony formation capability under the treatments with Pac.

To further confirm the existence of a signaling feedback loop, we examined whether specific AKT signaling inhibitors can sensitize the effects of targeting p38γ in model cells. Our data clearly showed that for 4T1 cells with p38γ MAPK knockdown, the 1 μM AKT inhibitor significantly decreased cell proliferation (Figure 5*B*). We also showed that the 0.5 and 1 μM AKT inhibitor significantly decreased colony formation in 4T1 cells with p38γ MAPK knockdown compared with 4T1 cells with vector control (Figure 5*C*). Similarly, two different doses of the specific PI3K inhibitor LY294002 (3 and 10 μM) and the Src inhibitor (25 and 50 μM) significantly inhibited the cell proliferation and colony formation in 4T1/p38γ-sh1 cells compared with 4T1 vector cells (Figure 5, *D-G*).

p38γ MAPK Mediates Cell Sensitivity to the PARP Inhibitor but Not to Two Conventional Chemotherapeutic Agents

To investigate whether mammary epithelial cells with p38γ MAPK expression acquire chemoresistant properties like many tumorigenic and metastatic contributors such as Snail, twist, and MTDH/AEG-1 [27–29], two conventional chemotherapeutic agents Dox and Pac were

used to treat several stable cell lines we established. As measured by MTT assay, we did not observe any changes in survival cell number in 67NR, MCF7, HMLE, and BT20 cells when treated with different doses of Dox and Pac (Figures 6, W10, W11, and W12). This result was also confirmed by the unchanged survival colony numbers measured with the clonogenic assay. In addition, Dox and Pac treatment in 4T1 cells with p38γ MAPK knockdown did not show any effect compared with 4T1 cells with vector control (data not shown). These data suggest that p38γ MAPK expression does not contribute to the resistance of regular chemotherapeutic agents in breast cancer cell lines.

We next studied whether the expression of p38γ MAPK contributes to cells' resistance of the PARP inhibitor, a recently developed therapeutic approach for cancer with BRCA mutation and basal-like breast cancer [30–32]. 67NR cells with ectopic expression of p38γ MAPK

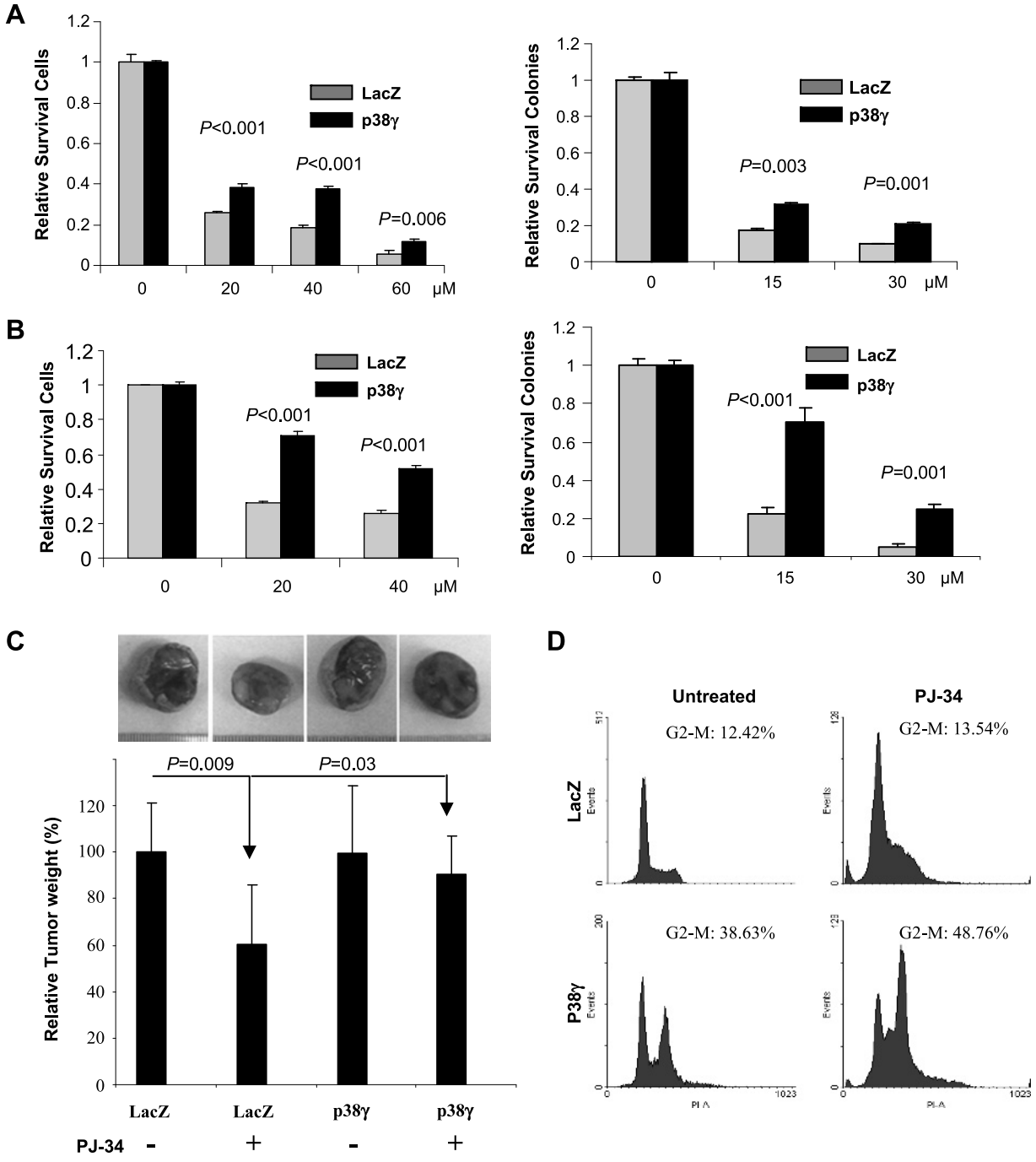


Figure 7. Effect of ectopic expression of p38γ MAPK on cell resistance to the PARP inhibitor. (A and B) The 67NR cells (A) and HMLE cells (B) with ectopic expression of p38γ MAPK show significant resistance to PARP inhibitor PJ-34. The left panel shows the cell proliferation after PJ-34 treatment. The right panel shows the quantification of the clonogenic assay results. Different doses of PJ-34 are shown. (C) Effect of p38γ MAPK and PJ-34 treatment on tumorigenesis *in vivo*. Relative final tumor weights were shown after PJ-34 was used for 16 days. (D) Ectopic expression p38γ MAPK and PJ-34 treatment led to G_2/M arrest in 67NR cells. 67NR cells with LacZ and p38γ MAPK expression were treated with DMSO (Untreated) and 15 μM PJ-34 for 2 days. Cell cycle was measured by flow cytometry assay.

and its vector control counterpart were treated with three different doses of PARP inhibitor PJ-34. We observed that significant cell survival and colony survival in 67NR cells with p38γ MAPK expression occurred compared with vector control cells (Figures 7*A* and W13). In addition, when HMLE-derived cells were treated with different doses of PJ-34, we also observed that HMLE cells with p38γ MAPK expression have significant survival rates compared with HMLE cells with vector control (Figures 7*B* and W14*A*). Similar results were also obtained when MCF7 cells were treated with different doses of PJ-34 (Figure W14*B*). Moreover, PJ-34 treatment on 4T1 cells with p38γ MAPK knockdown showed inhibitory effects on cell proliferation in a concentration higher than 15 μM (Figure W15*A*). The inhibitory effects of knockdown of p38γ MAPK and PJ-34 treatment were observed at a lower concentration at 2.5 μM when AKT inhibitor is added (Figure W15*B*). These data further confirmed the existence of the feed back loop of survival signaling. Furthermore, 67NR cells with ectopic expression of p38γ MAPK and its vector control counterpart were subcutaneously injected into nude mice. Without drug treatment, the 67NR cells with ectopic expression of p38γ MAPK and its vector control grew at equal rates. However, with the PJ-34 treatment, the tumor burden in 67NR cells with vector control group significantly shrank approximately 40% compared with untreated 67NR cells with vector control ($P = .009$). Significance was also observed when vector control group was compared with the p38γ MAPK expression group at day 16 after PJ-34 treatment (Figure 7*C*, $P = .03$).

Finally, we investigated the effect of p38γ MAPK on cell cycle. Ectopic expression of p38γ MAPK in 67NR cells induced clear G_2/M arrest (threefolds, 38.6%/12.42%). The G_2/M arrest was markedly enhanced when 67NR cells with p38γ expression were treated with PJ-34 (Figure 7*D*, 48.76%/38.63%). In contrast, no significant increase of G_2/M arrest was observed when the control 67NR cells were treated with PJ-34 (13.54%/12.42%), suggesting that p38γ MAPK overexpression is important to cell cycle arrest, and it can be the mechanism of breast cancer cells to survival in the DNA-damaging conditions.

Discussion

The results of our current study show specific overexpression of p38γ MAPK in basal-like breast cancer cell lines and tumor samples and correlate with the metastatic potential in breast tumor samples. These data are in agreement with several previous reports that indicated the potential oncogenic role of p38γ MAPK in cancer progression. However, a functional study showed that ectopic expression of p38γ MAPK in several breast cancer cell lines did not lead to significant changes in oncogenic characteristics. Conversely, knockdown of p38γ MAPK resulted in the significant diminution of the oncogenic characteristics of breast cancer cells, suggesting that the main function of p38γ MAPK in breast cancer is to maintain the oncogenic properties rather than to promote cancer progression. Other genetic alterations are needed to cooperate with p38γ MAPK to ensure complete tumorigenesis and metastasis of breast cancer cells.

Using the ectopic and knockdown model cell lines, our results demonstrated that p38γ MAPK is involved in the Ras/ERK signaling pathway in breast cancer. One interesting finding of the current study is that knockdown of p38γ MAPK in breast cancer cells led to the activation of the AKT signaling pathway, indicating the existence of a signaling feedback loop. From the therapeutic point of view, the signaling feedback loop is one reason of therapeutic resistance. Targeting the potential signaling feed back loop could significantly improve therapeutic efficacy [25,33–35]. Indeed, significant effects were observed when we treated p38γ MAPK knockdown breast cancer cells with various inhibitors targeting AKT signaling pathway. These data are supported by a report that shows that the combination of PI3K and MEK inhibitors confers high effectiveness in murine models of lung cancers that expressed mutant K-*ras* [36]. Moreover, the existence of the signaling feedback loop also conceals the effect of other therapeutic drugs such as PJ-34 (Figure W15). Taken together, the results of our study suggest that concomitant inhibition of AKT signaling with specific targeting p38γ MAPK could be a much more effective approach than only targeting p38γ MAPK in breast cancer treatment.

It has been shown that cells deficient in DNA DSB repair, in particular homologous recombination (HR) by gene conversion, are highly sensitive to chemical inhibitions of PARP [30,31,37]. Conversely, the breast and ovarian cancer predisposition genes, *BRCA1* and *BRCA2*, encode proteins that are required for efficient HR [38,39]. Therefore, PARP inhibitor treatment could be an effective approach to target cancers with *BRCA1* and *BRCA2* mutations, which are HR-defective. Moreover, basal-like or triple-negative breast cancers have features that mimic those of tumors arising in *BRCA1* mutation carriers. Most patients with *BRCA1* germ line mutations develop basal-like or triple-negative breast cancers [40–42]. This overlap suggests that part of the basal-like or triple-negative breast cancers, for which targeted therapy is unavailable, may respond to therapeutics targeting *BRCA1*-deficient cells [43]. Our current study showed that p38γ MAPK up-regulation induced a significant resistance to PARP inhibitor treatment in breast cancer cell lines. These data are consistent with a previous report that shows that p38γ MAPK is one of the six genes mediating sensitivity to the PARP inhibitor by a synthetic lethal small interfering RNA screen [44]. Moreover, ectopic expression of p38γ MAPK could lead to significant cell cycle arrest in the G_2/M phase. Taken together, our data indicate that the p38γ MAPK is a negative mediator of the cellular sensitivity to DNA damage in breast cancer. Targeting p38γ MAPK along with PARP treatment could increase the therapeutic efficacy in the basal-like breast cancer population.

In summary, our study for the first time provides solid biologic evidences implicating p38γ MAPK as a critical molecule for maintaining oncogenic properties and contributing to specific resistance to the PARP inhibitor in basal-like breast cancer. Future studies that will include the design of specific p38γ MAPK inhibitor, the generation of transgenic or knockout mice, and the further exploration of the mechanism underlying p38γ MAPK are needed to eventually establish the role of p38γ MAPK as a therapeutic candidate for of breast cancer treatment.

Acknowledgments

The authors thank Stephen P. Ethier of Karmanos Cancer Institute, Wayne State University, and Robert A. Weinberg of Massachusetts Institute of Technology for providing technical advice and agents. The authors also thank Elizabeth A. Katz of the Barbara Ann Karmanos Cancer Institute's Marketing & Communications Department for editing our article.

References

[1] Cuenda A and Rousseau S (2007). p38 MAP-kinases pathway regulation, function and role in human diseases. *Biochim Biophys Acta* **1773**, 1358–1375.

[2] Dhanasekaran DN and Johnson GL (2007). MAPKs: function, regulation, role in cancer and therapeutic targeting. *Oncogene* **26**, 3097–3099.

[3] Goedert M, Cuenda A, Craxton M, Jakes R, and Cohen P (1997). Activation of the novel stress-activated protein kinase SAPK4 by cytokines and cellular stresses

is mediated by SKK3 (MKK6); comparison of its substrate specificity with that of other SAP kinases. *EMBO J* **16**, 3563–3571.
[4] Han J, Lee JD, Bibbs L, and Ulevitch RJ (1994). A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* **265**, 808–811.
[5] Jiang Y, Chen C, Li Z, Guo W, Gegner JA, Lin S, and Han J (1996). Characterization of the structure and function of a new mitogen-activated protein kinase (p38β). *J Biol Chem* **271**, 17920–17926.
[6] Jiang Y, Gram H, Zhao M, New L, Gu J, Feng L, Di Padova F, Ulevitch RJ, and Han J (1997). Characterization of the structure and function of the fourth member of p38 group mitogen-activated protein kinases, p38δ. *J Biol Chem* **272**, 30122–30128.
[7] Lechner C, Zahalka MA, Giot JF, Moller NP, and Ullrich A (1996). ERK6, a mitogen-activated protein kinase involved in C2C12 myoblast differentiation. *Proc Natl Acad Sci USA* **93**, 4355–4359.
[8] Freshney NW, Rawlinson L, Guesdon F, Jones E, Cowley S, Hsuan J, and Saklatvala J (1994). Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. *Cell* **78**, 1039–1049.
[9] Rouse J, Cohen P, Trigon S, Morange M, Alonso-Llamazares A, Zamanillo D, Hunt T, and Nebreda AR (1994). A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell* **78**, 1027–1037.
[10] Conrad PW, Rust RT, Han J, Millhorn DE, and Beitner-Johnson D (1999). Selective activation of p38α and p38γ by hypoxia. Role in regulation of cyclin D1 by hypoxia in PC12 cells. *J Biol Chem* **274**, 23570–23576.
[11] Pramanik R, Qi X, Borowicz S, Choubey D, Schultz RM, Han J, and Chen G (2003). p38 isoforms have opposite effects on AP-1–dependent transcription through regulation of c-Jun. The determinant roles of the isoforms in the p38 MAPK signal specificity. *J Biol Chem* **278**, 4831–4839.
[12] Wang L, Ma R, Flavell RA, and Choi ME (2002). Requirement of mitogen-activated protein kinase kinase 3 (MKK3) for activation of p38α and p38δ MAPK isoforms by TGF-β_1 in murine mesangial cells. *J Biol Chem* **277**, 47257–47262.
[13] Li Z, Jiang Y, Ulevitch RJ, and Han J (1996). The primary structure of p38γ: a new member of p38 group of MAP kinases. *Biochem Biophys Res Commun* **228**, 334–340.
[14] Abdollahi T, Robertson NM, Abdollahi A, and Litwack G (2003). Identification of interleukin 8 as an inhibitor of tumor necrosis factor–related apoptosis-inducing ligand-induced apoptosis in the ovarian carcinoma cell line OVCAR3. *Cancer Res* **63**, 4521–4526.
[15] Liu LX, Liu ZH, Jiang HC, Zhang WH, Qi SY, Hu J, Wang XQ, and Wu M (2003). Gene expression profiles of hepatoma cell line HLE. *World J Gastroenterol* **9**, 683–687.
[16] Tang J, Qi X, Mercola D, Han J, and Chen G (2005). Essential role of p38γ in K-Ras transformation independent of phosphorylation. *J Biol Chem* **280**, 23910–23917.
[17] Wang X, McGowan CH, Zhao M, He L, Downey JS, Fearns C, Wang Y, Huang S, and Han J (2000). Involvement of the MKK6-p38γ cascade in γ-radiation–induced cell cycle arrest. *Mol Cell Biol* **20**, 4543–4552.
[18] Sakabe K, Teramoto H, Zohar M, Behbahani B, Miyazaki H, Chikumi H, and Gutkind JS (2002). Potent transforming activity of the small GTP-binding protein Rit in NIH 3T3 cells: evidence for a role of a p38γ-dependent signaling pathway. *FEBS Lett* **511**, 15–20.
[19] Qi X, Tang J, Loesch M, Pohl N, Alkan S, and Chen G (2006). p38γ mitogen-activated protein kinase integrates signaling crosstalk between Ras and estrogen receptor to increase breast cancer invasion. *Cancer Res* **66**, 7540–7547.
[20] Loesch M, Zhi HY, Hou SW, Qi XM, Li RS, Basir Z, Iftner T, Cuenda A, and Chen G (2010). p38γ MAPK cooperates with c-Jun in trans-activating matrix metalloproteinase 9. *J Biol Chem* **285**, 15149–15158.
[21] Kwong J, Hong L, Liao R, Deng Q, Han J, and Sun P (2009). p38α and p38γ mediate oncogenic *ras*-induced senescence through differential mechanisms. *J Biol Chem* **284**, 11237–11246.
[22] Aslakson CJ and Miller FR (1992). Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. *Cancer Res* **52**, 1399–1405.
[23] Morel AP, Lievre M, Thomas C, Hinkal G, Ansieau S, and Puisieux A (2008). Generation of breast cancer stem cells through epithelial-mesenchymal transition. *PLoS One* **3**, e2888.
[24] Mani SA, Yang J, Brooks M, Schwaninger G, Zhou A, Miura N, Kutok JL, Hartwell K, Richardson AL, and Weinberg RA (2007). Mesenchyme Forkhead 1 (FOXC2) plays a key role in metastasis and is associated with aggressive basal-like breast cancers. *Proc Natl Acad Sci USA* **104**, 10069–10074.
[25] Zhang H, Chen D, Ringler J, Chen W, Cui QC, Ethier SP, Dou QP, and Wu G (2010). Disulfiram treatment facilitates phosphoinositide 3-kinase inhibition in human breast cancer cells *in vitro* and *in vivo*. *Cancer Res* **70**, 3996–4004.
[26] Zhang H, Liu G, Dziubinski M, Yang Z, Ethier SP, and Wu G (2008). Comprehensive analysis of oncogenic effects of PIK3CA mutations in human mammary epithelial cells. *Breast Cancer Res Treat* **112**, 217–227.
[27] Hu G, Chong RA, Yang Q, Wei Y, Blanco MA, Li F, Reiss M, Au JL, Haffty BG, and Kang Y (2009). MTDH activation by 8q22 genomic gain promotes chemoresistance and metastasis of poor-prognosis breast cancer. *Cancer Cell* **15**, 9–20.
[28] Su HY, Lai HC, Lin YW, Liu CY, Chen CK, Chou YC, Lin SP, Lin WC, Lee HY, and Yu MH (2010). Epigenetic silencing of SFRP5 is related to malignant phenotype and chemoresistance of ovarian cancer through Wnt signaling pathway. *Int J Cancer* **127**, 555–567.
[29] Zhuo WL, Wang Y, Zhuo XL, Zhang YS, and Chen ZT (2008). Short interfering RNA directed against TWIST, a novel zinc finger transcription factor, increases A549 cell sensitivity to cisplatin via MAPK/mitochondrial pathway. *Biochem Biophys Res Commun* **369**, 1098–1102.
[30] Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, Kyle S, Meuth M, Curtin NJ, and Helleday T (2005). Specific killing of *BRCA2*-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* **434**, 913–917.
[31] Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, Santarosa M, Dillon KJ, Hickson I, Knights C, et al. (2005). Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* **434**, 917–921.
[32] Fong PC, Yap TA, Boss DS, Carden CP, Mergui-Roelvink M, Gourley C, De Greve J, Lubinski J, Shanley S, Messiou C, et al. (2010). Poly(ADP)-ribose polymerase inhibition: frequent durable responses in BRCA carrier ovarian cancer correlating with platinum-free interval. *J Clin Oncol* **28**, 2512–2519.
[33] Engelman JA (2009). Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer* **9**, 550–562.
[34] O'Reilly KE, Rojo F, She QB, Solit D, Mills GB, Smith D, Lane H, Hofmann F, Hicklin DJ, Ludwig DL, et al. (2006). mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res* **66**, 1500–1508.
[35] Wu G (2010). Recent progress in phosphoinositide 3-kinases: oncogenic properties and prognostic and therapeutic implications. *Curr Protein Pept Sci* **11**, 425–435.
[36] Engelman JA, Chen L, Tan X, Crosby K, Guimaraes AR, Upadhyay R, Maira M, McNamara K, Perera SA, Song Y, et al. (2008). Effective use of PI3K and MEK inhibitors to treat mutant *Kras* G12D and PIK3CA H1047R murine lung cancers. *Nat Med* **14**, 1351–1356.
[37] McCabe N, Turner NC, Lord CJ, Kluzek K, Bialkowska A, Swift S, Giavara S, O'Connor MJ, Tutt AN, Zdzienicka MZ, et al. (2006). Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly(ADP-ribose) polymerase inhibition. *Cancer Res* **66**, 8109–8115.
[38] Moynahan ME, Chiu JW, Koller BH, and Jasin M (1999). *Brca1* controls homology-directed DNA repair. *Mol Cell* **4**, 511–518.
[39] Tutt A, Bertwistle D, Valentine J, Gabriel A, Swift S, Ross G, Griffin C, Thacker J, and Ashworth A (2001). Mutation in *Brca2* stimulates error-prone homology-directed repair of DNA double-strand breaks occurring between repeated sequences. *EMBO J* **20**, 4704–4716.
[40] Miyoshi Y, Murase K, and Oh K (2008). Basal-like subtype and *BRCA1* dysfunction in breast cancers. *Int J Clin Oncol* **13**, 395–400.
[41] Rakha EA, Reis-Filho JS, and Ellis IO (2008). Basal-like breast cancer: a critical review. *J Clin Oncol* **26**, 2568–2581.
[42] Tischkowitz MD and Foulkes WD (2006). The basal phenotype of *BRCA1*-related breast cancer: past, present and future. *Cell Cycle* **5**, 963–967.
[43] Jaspers JE, Rottenberg S, and Jonkers J (2009). Therapeutic options for triple-negative breast cancers with defective homologous recombination. *Biochim Biophys Acta* **1796**, 266–280.
[44] Turner NC, Lord CJ, Iorns E, Brough R, Swift S, Elliott R, Rayter S, Tutt AN, and Ashworth A (2008). A synthetic lethal siRNA screen identifying genes mediating sensitivity to a PARP inhibitor. *EMBO J* **27**, 1368–1377.

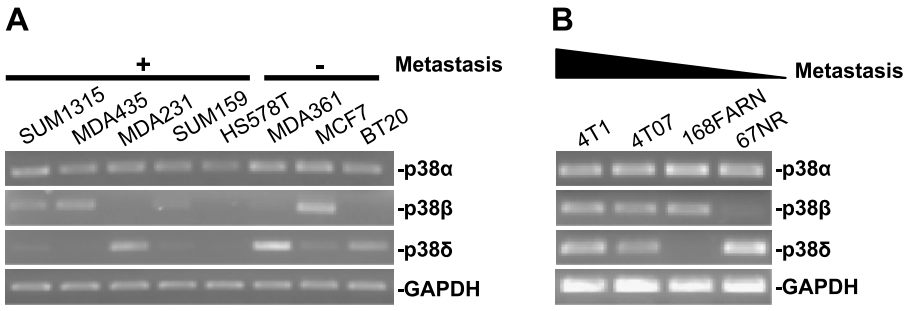
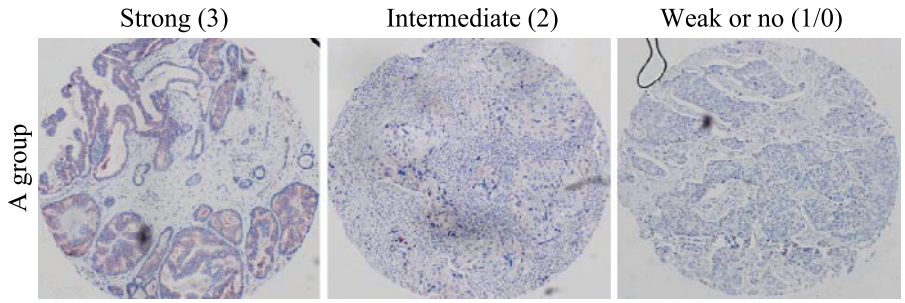


Figure W1. The expression pattern of different p38 MAPKs in breast cancer cell lines. (A) Expression of p38α, p38β, and p38δ MAPK in a panel of human breast cancer cell lines. "+" stands for highly metastatic cell lines. "−" stands for poorly metastatic cell lines. GAPDH is an RNA loading control. (B) Expression of p38α, p38β, and p38δ MAPK in a panel of mouse breast cancer cell lines. Triangular bar indicates the increase of metastasis capability. GAPDH is an RNA loading control.

A



B

	strong	intermediate	weak/no	Total	raw p values
ER positive	7	9	18	34	0.8459
ER negative	8	7	21	36	
total	15	16	39	70	
Ki67 positive	10	12	22	44	0.3452
Ki67 negative	5	4	17	26	
total	15	16	39	70	
EGFR positive	6	2	6	14	0.07611
EGFR negative	9	14	33	56	
total	15	16	39	70	
HER2 positive	12	8	22	42	0.1843
HER2 negative	3	8	17	28	
total	15	16	39	70	
AR positive	9	10	9	28	0.003774
AR negative	6	6	30	42	
total	15	16	39	70	
PR positive	5	8	12	25	0.6279
PR negative	10	8	27	45	
total	15	16	39	70	

Figure W2. IHC results in a tissue microarray. (A) Representative IHC staining figure for breast tumor samples with lymph node and distant metastasis. (B) Statistical analysis of the relation of p38γ expression and different pathologic characteristics of breast tumor samples; five normal samples were excluded from these analyses.

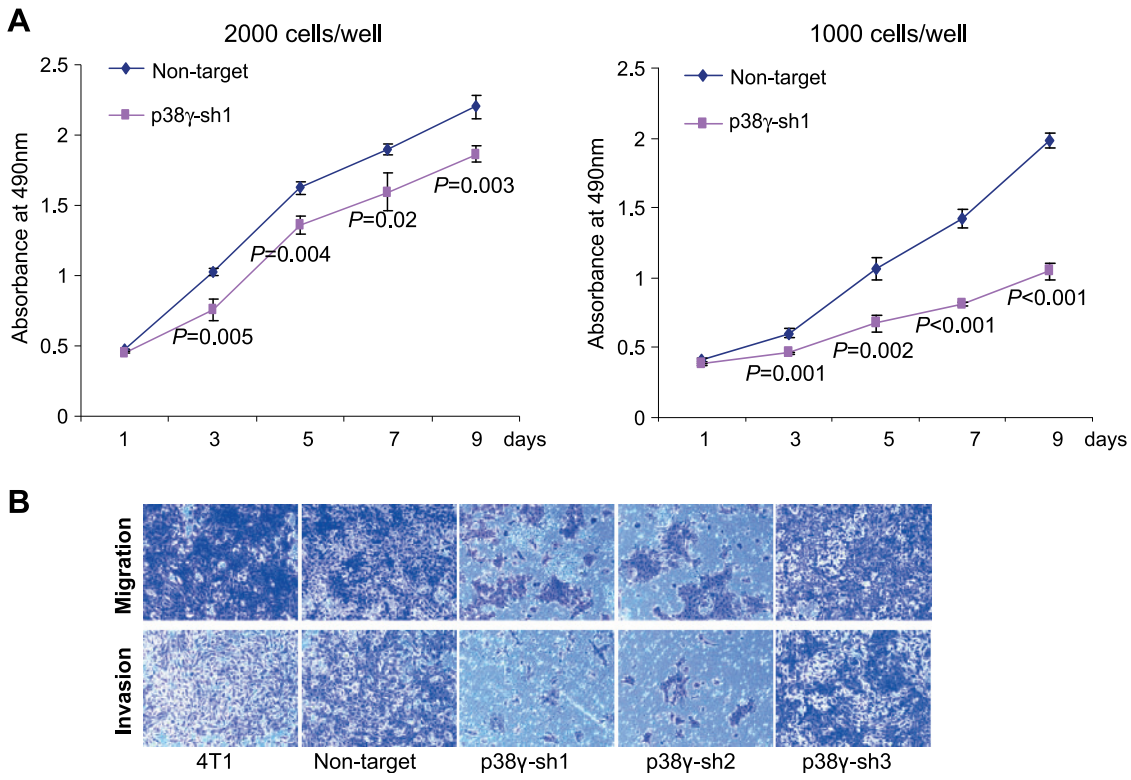


Figure W3. Effect of knockdown of p38γ MAPK on cell proliferation, migration, and invasion *in vitro*. (A) Comparison of effect of different cell numbers on cell proliferation. Cells (2000 cells/well [left panel] and 1000 cells/well [right panel]) were seeded in 96-well plates and MTT assays were tested. (B) Representative figures show the migration (top) and invasion (bottom) abilities of 4T1 parental cells, 4T1 cells transfected with nontarget vector, and three shRNA clones.

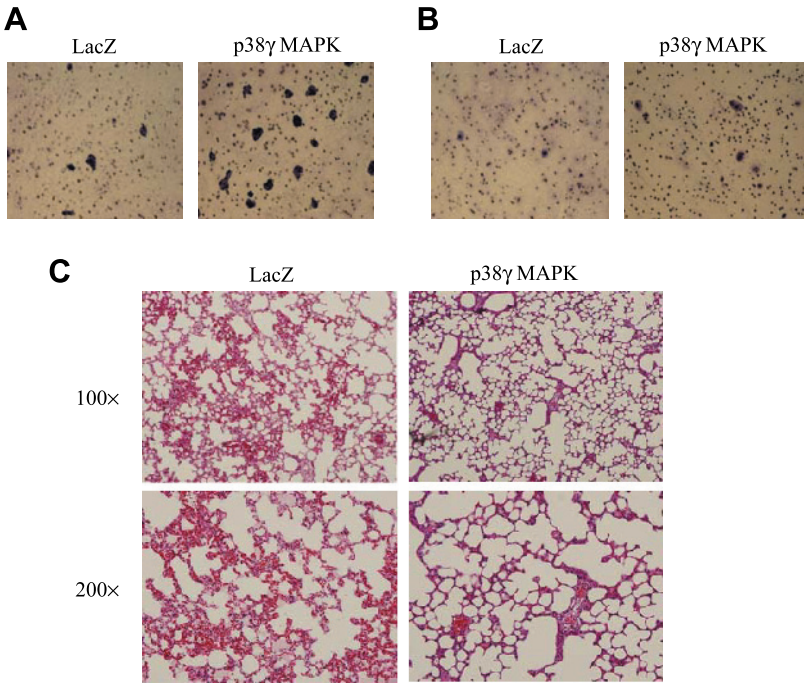


Figure W4. Effect of ectopic expression of p38γ MAPK on migration, invasion, and metastasis in MCF7 cells. (A) Representative figures for the migration assay using MCF7/LacZ and MCF7/p38γ MAPK cells. (B) Representative figures for the invasion assay using MCF7/LacZ and MCF7/p38γ MAPK cells. (C) Representative figure for the lung section from the mice xenografted with MCF7/LacZ and MCF7/p38γ MAPK cells. No significant metastasis was observed in both xenograft models.

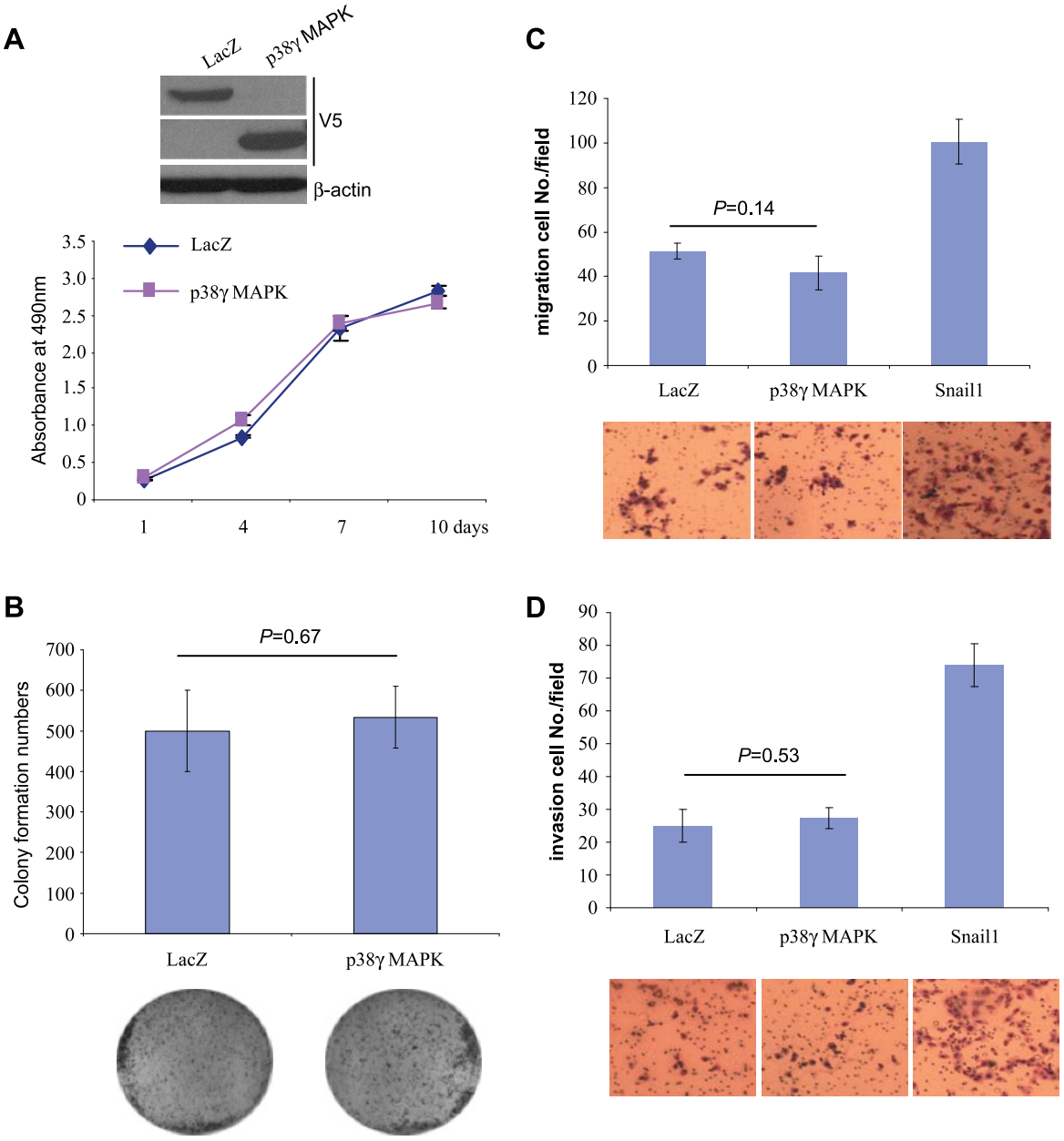


Figure W5. The effect of overexpression of p38γ MAPK on cell proliferation, colony formation, migration, and invasion in HMLE cells. (A) Top panel confirmed the expression of p38γ MAPK in HMLE cells. MTT assay shows no changes of cell proliferation when p38γ MAPK was overexpressed in HMLE cells (lower panel). (B) Colony formation assay shows that overexpression of p38γ MAPK in HMLE cells does not change the colony formation capability of HMLE cells ($P = .67$). (C and D) Overexpression of p38γ MAPK in HMLE cells does not affect cell migration (C; $P = .14$) and invasion (D; $P = .53$) capabilities.

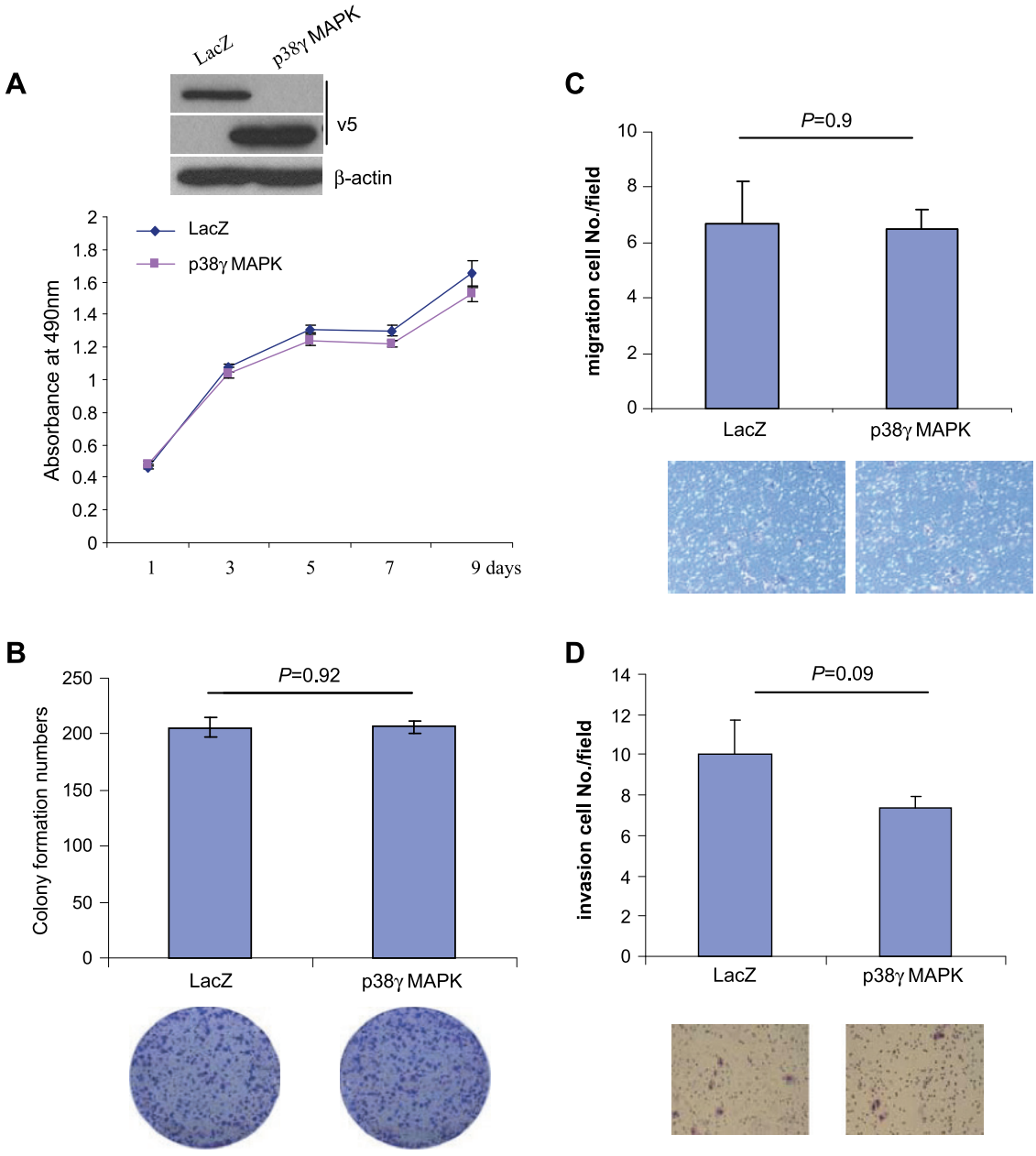


Figure W6. Effect of overexpression of p38γ MAPK on cell proliferation, colony formation, migration, and invasion in BT20 cells. (A) Top panel shows the ectopic expression of p38γ MAPK in BT20 cells. MTT assay shows no changes of cell proliferation when p38γ MAPK was overexpressed in BT20 cells (lower panel). (B) Colony formation assay shows that overexpression of p38γ MAPK in BT20 cells does not change the colony formation capability of BT20 cells ($P = .92$). (C and D) Overexpression of p38γ MAPK in BT20 cells does not affect cell migration (C; $P = .90$) and invasion (D; $P = .66$) capabilities.

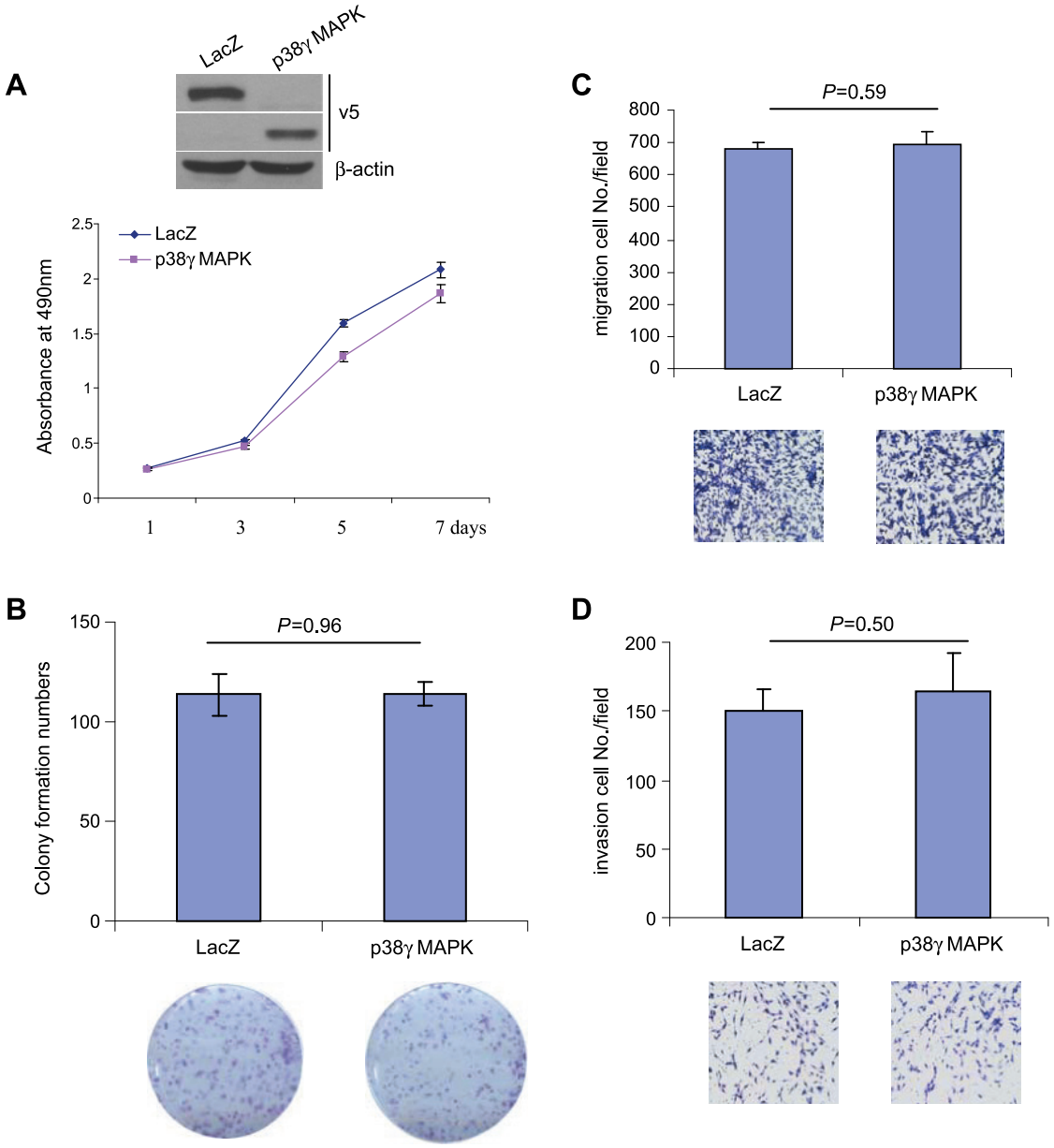


Figure W7. Effect of overexpression of p38γ MAPK on cell proliferation, colony formation, migration and invasion in 67NR cells. (A) Top panel shows the ectopic expression of p38γ MAPK in 67NR cells. MTT assay shows no changes in cell proliferation when p38γ MAPK was overexpressed in 67NR cells. (B) Colony formation assay shows that overexpression of p38γ MAPK in 67NR cells does not change the colony formation capability of 67NR cells ($P = .96$). (C and D) Overexpression of p38γ MAPK in 67NR cells does not affect cell migration (C; $P = .59$) and invasion (D; $P = .50$) capabilities.

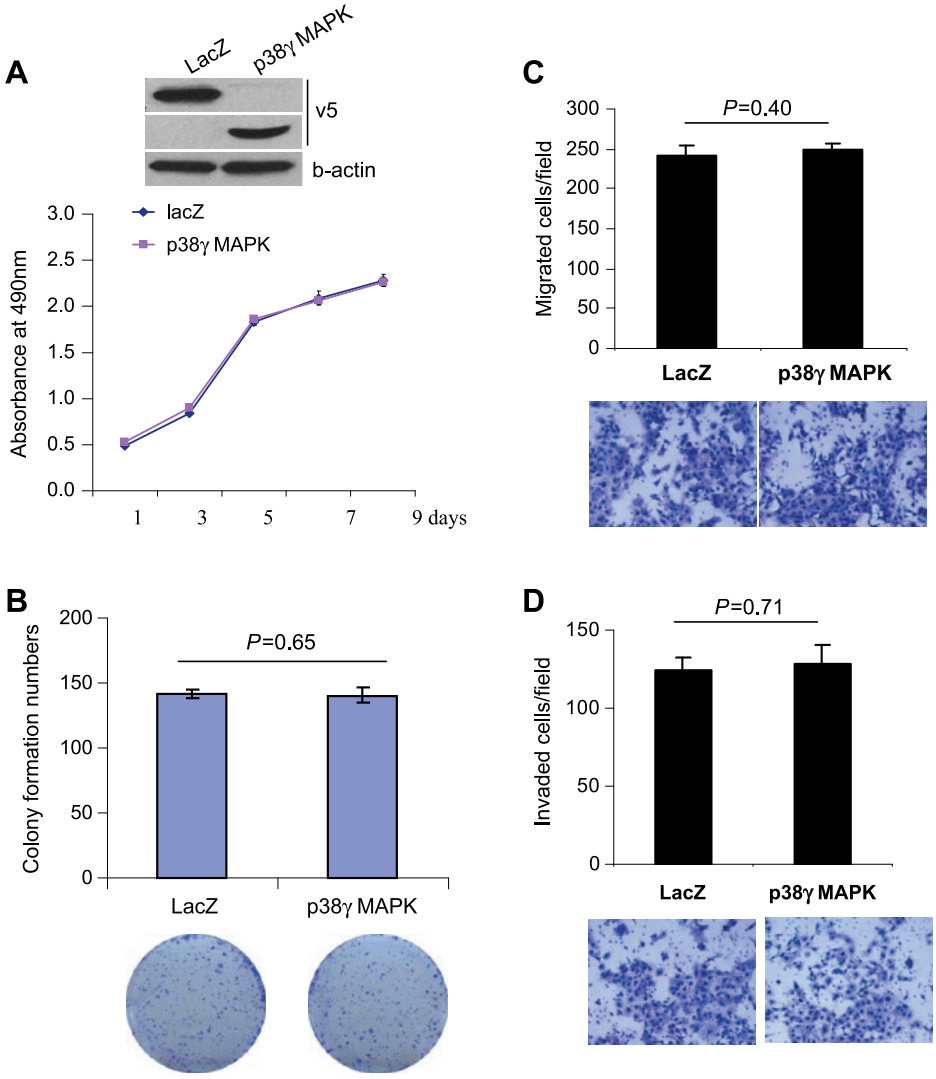


Figure W8. Effects of overexpression of p38γ MAPK on cell proliferation, colony formation, and invasion in EpRas cells. (A) Top panel shows ectopic expression of p38γ MAPK in EpRas cells. MTT assay shows no changes of cell proliferation when p38γ MAPK was overexpressed in EpRas cells (lower panel). (B) Colony formation assay shows that overexpression of p38γ MAPK in EpRas cells does not change the colony formation capability of EpRas cells ($P = .65$). (C and D) Overexpression of p38γ MAPK in EpRas cells does not affect cell migration (C; $P = .40$) and invasion (D; $P = .71$) capabilities.

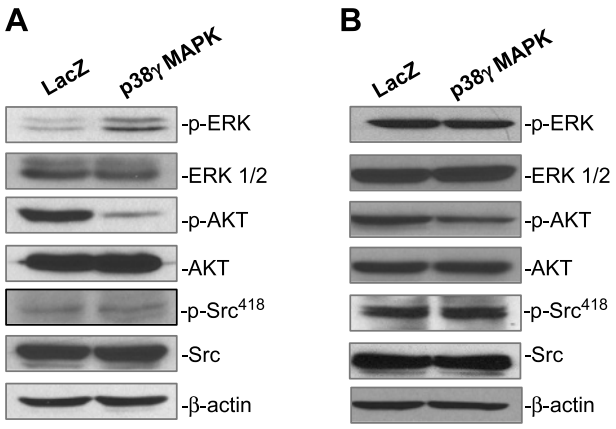


Figure W9. Effect of p38γ MAPK on cell signaling pathways in p38γ MAPK overexpressed cells. (A) Effect of p38γ MAPK on AKT and ERK signaling in EpRas cells. (B) Effect of p38γ MAPK on AKT and ERK signaling in MCF7 cells.

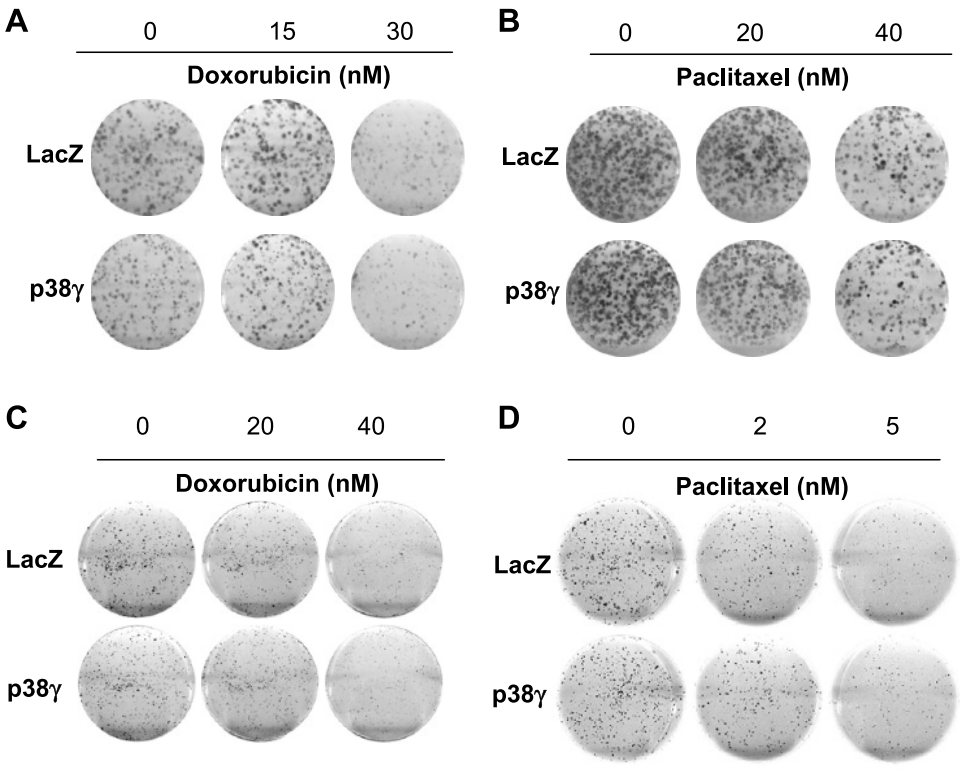


Figure W10. Effects of p38γ MAPK overexpression on chemoresistance in 67NR cells and MCF7 cells. (A and B) Representative colony formation plates showing the effect of treatment of different doses of two chemotherapeutic agents Dox and Pac on 67NR cells. (C and D) Representative colony formation plates showing the effect of treatment of different doses of two chemotherapeutic agents Dox and Pac on MCF7 cells.

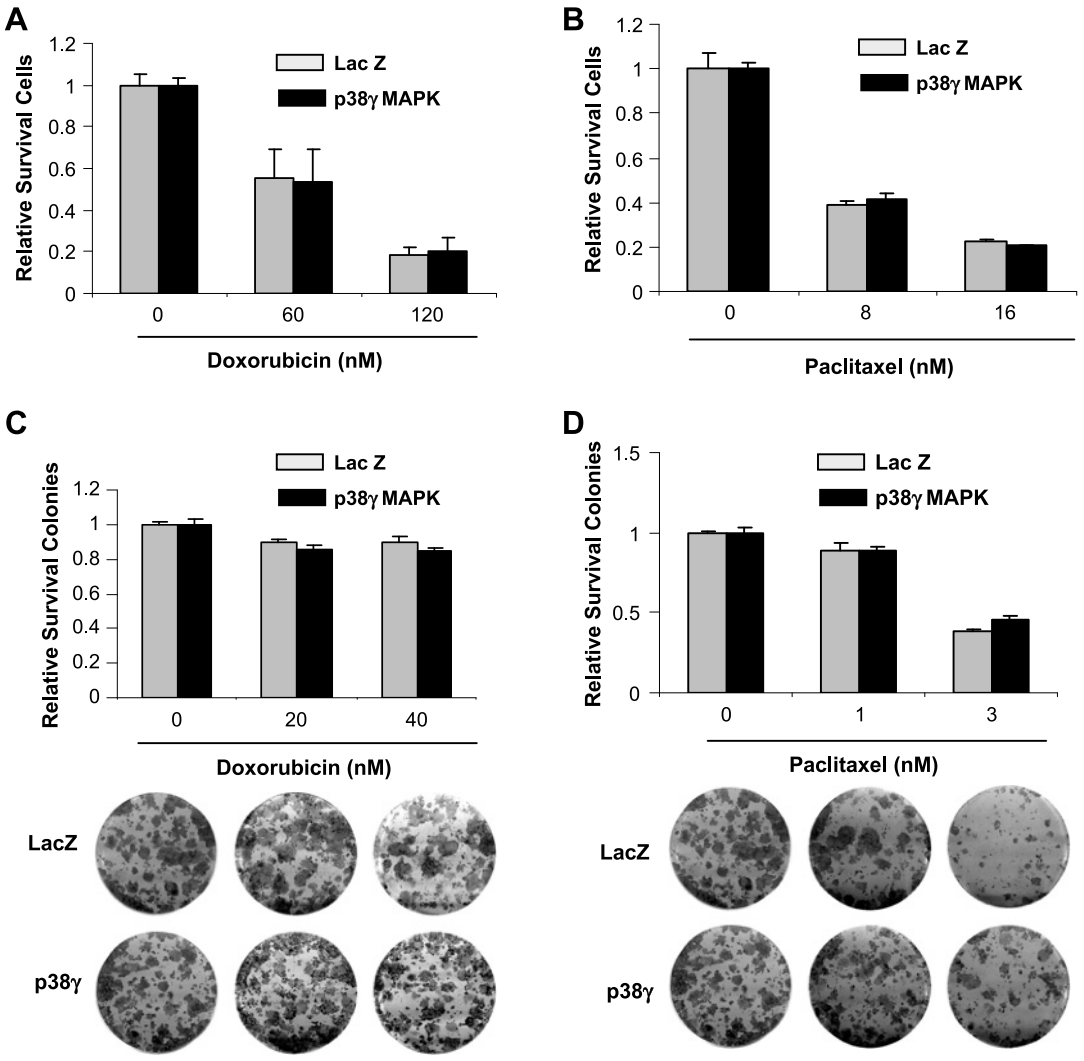


Figure W11. Effects of Dox and Pac on HMLE cells with or without p38γ MAPK overexpression. (A) Overexpression of p38γ MAPK in HMLE cells has no effect on the cell proliferation under the treatments with Dox. (B) Overexpression of p38γ MAPK in HMLE cells has no effect on the cell proliferation under the treatments with Pac. (C) Overexpression of p38γ MAPK in HMLE cells has no effect on the colony formation capability under the treatments with Dox. (D) Overexpression of p38γ MAPK in HMLE cells has no effect to the colony formation capability under the treatments with Pac.

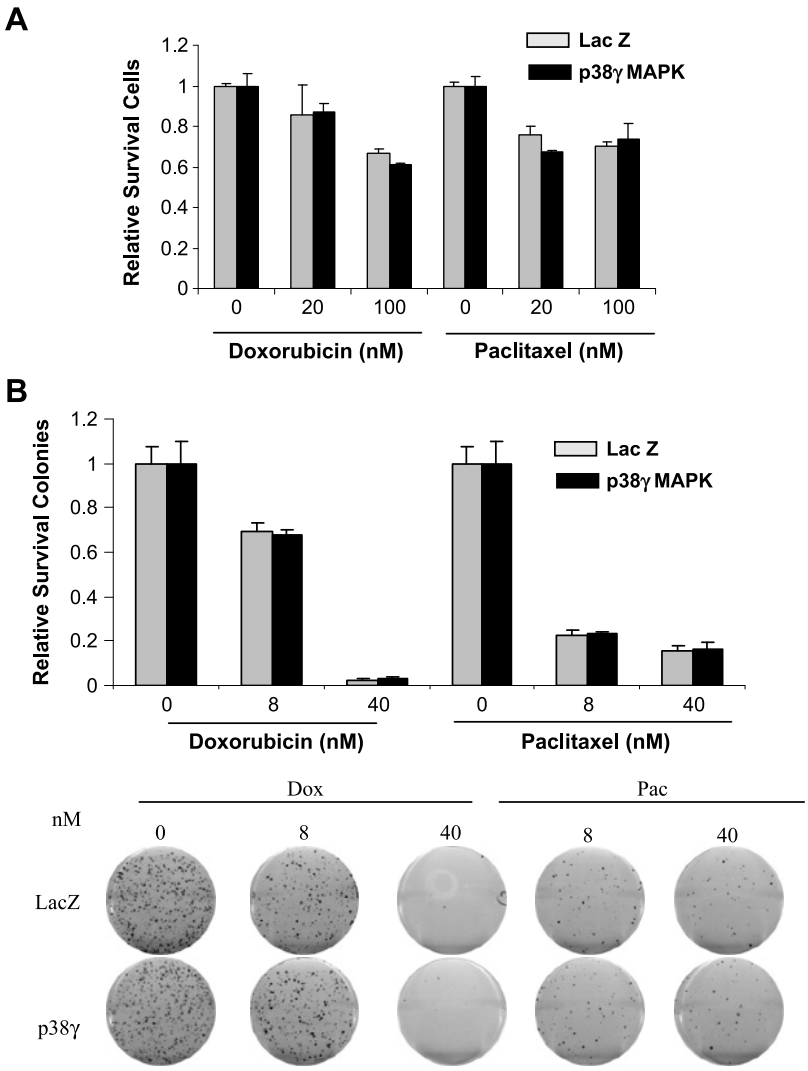


Figure W12. Effects of Dox and Pac on BT20 cells with or without p38γ MAPK overexpression. (A) Overexpression of p38γ MAPK in BT20 cells has no effect on the cell proliferation under the treatments with Dox and Pac. (B) Overexpression of p38γ MAPK in BT20 cells has no effect on the colony formation capability under the treatments with different doses of Dox and Pac. Top panel shows the relative survival colonies. Lower panels show the representative plates of colony formation.

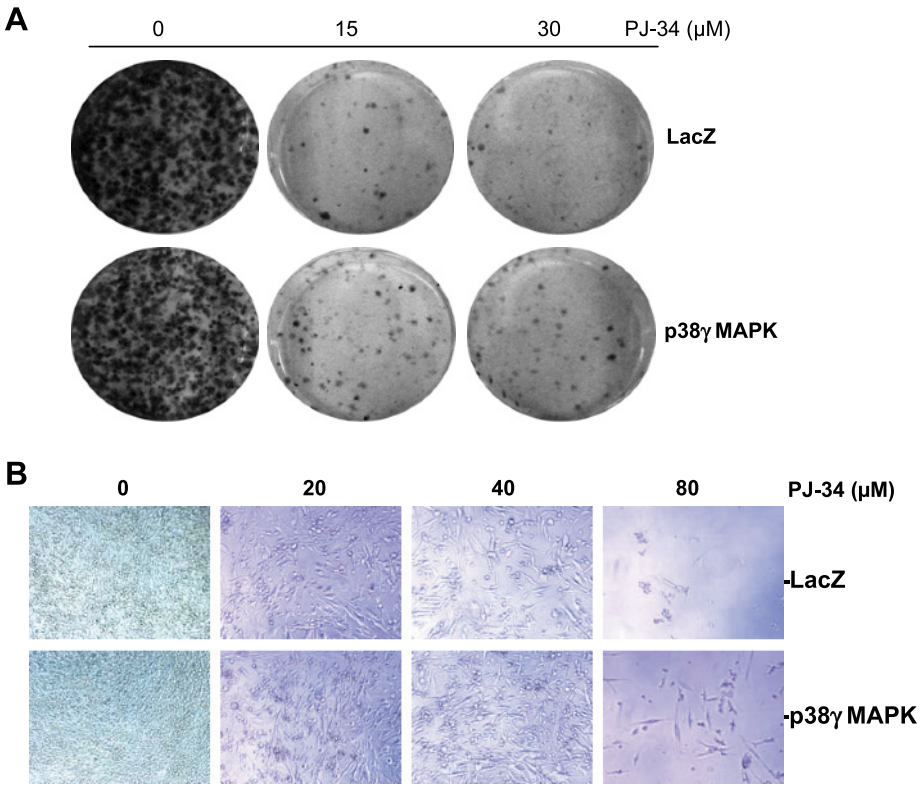


Figure W13. Effects of PJ-34 treatment on 67NR cells with or without p38γ MAPK overexpression. (A) Representative figures for the clonogenic assay for HMLE/Lac/Z and HMLE/p38γ MAPK treated with PJ-34. (B) Morphologic change of the 67NR cells when treated with different doses of PJ-34.

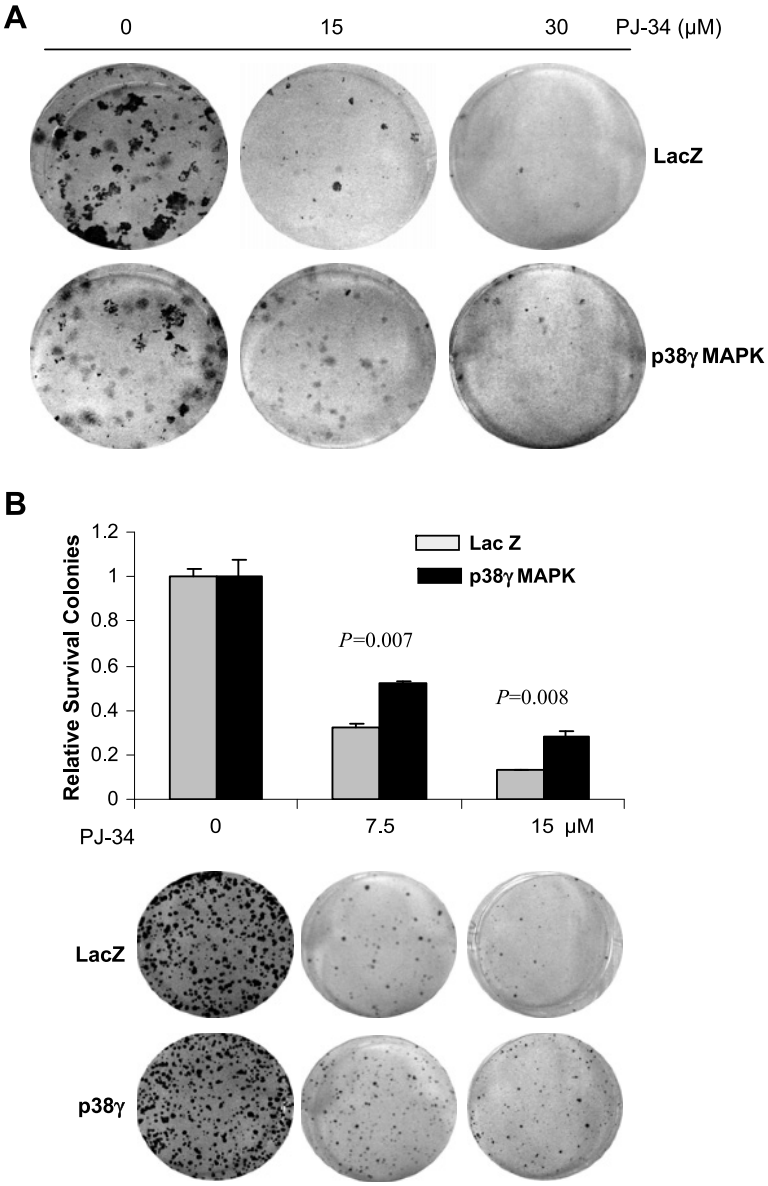


Figure W14. Effects of PJ-34 on HMLE (A) and MCF7 cells (B) with or without p38γ MAPK overexpression. (A) Representative figures for the colongenic assay for HMLE/Lac/Z and HMLE/p38γ MAPK treated with PJ34. (B) Overexpression of p38γ MAPK in MCF7 cells increases the resistance to PJ-34 in colony formation assays. Top panel shows the summary of the results from three independent assays. Lower panel shows the representative figures.

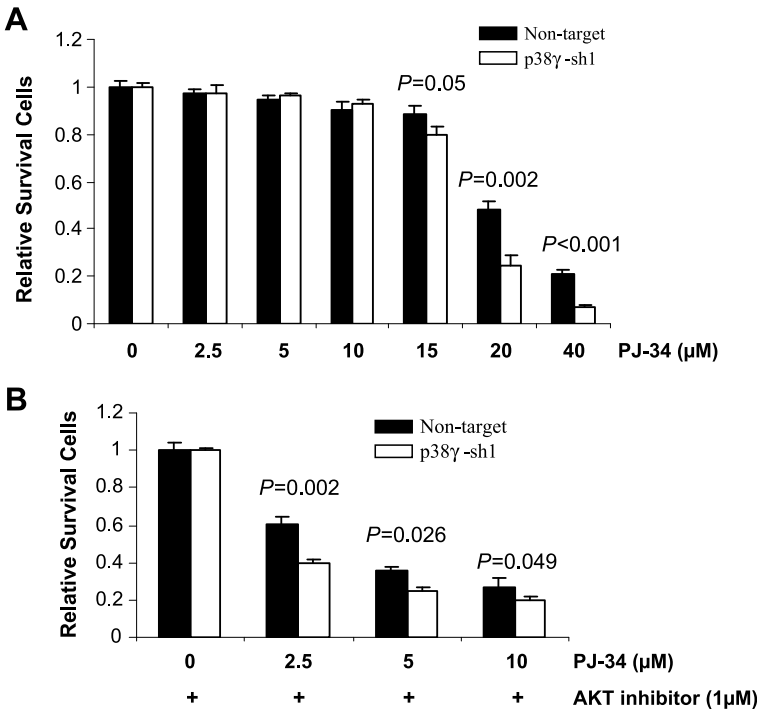


Figure W15. Effects of PJ-34 treatment and AKT inhibitor on in 4T1 cells with p38γ MAPK knockdown. (A) Survival cell number is significantly decreased in 4T1 cells with p38γ MAPK knockdown when the PJ-34 concentration is higher than 15 μm. (B) With the AKT inhibitor VIII (AKT-1/2, 1 μm), the survival cell number is significantly decreased in 4T1 cells with p38γ MAPK knockdown when the PJ-34 concentration is 2.5 μm.